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(54) Title: COLEOPTERAN ACTIVE MICROORGANISMS, RELATED INSECTICIDE COMPOSITIONS AND METHODS FOR THEIR PRODUCTION AND USE

(57) Abstract

This inventon relates to biologically pure cultures of *Bacillus thuringiensis* strains which have insecticidal activity at least against insects of the order Coleoptera. This invention also relates to the crystalline protein toxin useful as a biological insecticide against Coleoptera which toxin is produced by the strain of *Bacillus thuringiensis*. This invention also relates to the expression in various microorganisms of the gene, known as *cryC*, which codes for this toxin, and for related novel insecticide compositions and methods for their use.

COLEOPTERAN ACTIVE MICROORGANISMS, RELATED INSECTICIDE COMPOSITIONS AND METHODS FOR THEIR PRODUCTION AND USE

1.0 INTRODUCTION

5   This invention relates to biologically pure cultures of Bacillus thuringiensis strains which have insecticidal activity at least against insects of the order Coleoptera. This invention also relates to the crystalline protein toxin which is useful as a

10   biological insecticide against coleopteran insects. The toxin is naturally produced by this strain of Bacillus thuringiensis. This invention also relates to the expression in various microorganisms of the gene, herein referred to as cryC, which codes for the coleopteran

15   active toxin and for related novel insecticide compositions incorporating the toxin itself and microorganisms transformed with the cryC gene.

2.0 BACKGROUND OF THE INVENTION

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2.1 COMMERCIAL PESTICIDES: GENERAL CONSIDERATIONS

        Each year, significant portions of the world's commercially important agricultural crops are lost to insects and other pest infestation. The damage wrought

25   by these pests extends to all areas of commercially important plants including foods, textiles, and various domestic plants, and the economic damage runs well into the millions of dollars. Thus, protection of crops from such infestations is of paramount concern.

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        Broad spectrum pesticides are most commonly used for crop protection, but indiscriminate use of these agents can lead to disruption of the plant's natural defensive agents. Furthermore, because of their broad

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spectrum of activity, the chemical pesticides may destroy
non-target organisms such as beneficial insects and
parasites of destructive pests. These are also
frequently toxic to animals and humans and, thus, pose

5 environmental hazards when applied.

Additionally, insects and other organisms have
frequently developed resistance to these pesticides when
repeatedly exposed to them. In addition to reducing the

10 utility of the pesticide, resistant strains of minor
pests may become major infestation problems due to the
reduction of beneficial parasitic organisms.

This is a major problem encountered in using

15 broad spectrum pesticides. What is needed is a
biodegradable pesticide that combines a narrower spectrum
of activity with the ability to maintain its activity
over an extended period of time, i.e., to which
resistance develops much more slowly, or not at all.

20 Biopesticides appear to be useful in this regard.

2.2. BIOLOGICAL PESTICIDES

Biopesticides, also called biorationals, make

25 use of naturally occurring pathogens to control insects,
fungal, and weed infestations of agricultural crops.
Such substances may comprise a bacterium which produces a
substance toxic to the infesting agent (such as a toxin),
with or without a bacterial growth medium. Such

30 bacteria, which can be applied directly to the plants by
standard methods of application, are typically less
harmful to non-target organisms, and to the environment
as a whole, in comparison to chemical pesticides.

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The use of biological methods of pest control
was first suggested in 1895 when a fungal disease was
discovered in silkworms. It was not until 1940, however,
when spores of the milky disease bacterium <u>Bacillus</u>

5   <u>popilliae</u> were used to control the Japanese beetle, that
successful biological pest control was first achieved.
The bacterium, named <u>Bacillus</u> <u>thuringiensis</u> (hereinafter
referred to alternatively as "B.t."or "BT"), a bacteria
that produces a toxin fatal to caterpillars and other

10   insects, is currently the most widely used biopesticide.
In the late 1960's, the discovery of HD-1, a highly toxic
strain of B.t., set the stage for commercial use of
biopesticides.

15   2.3   <u>BACILLUS THURINGIENSIS AND DELTA-ENDOTOXINS</u>

<u>Bacillus</u> <u>thuringiensis</u> is a widely distributed,
rod shaped, aerobic, spore-forming microorganism. During
its sporulation cycle B.t. forms proteins known as

20   protoxins or delta-endotoxins. These protoxins are
deposited in B.t. as parasporal, crystalline inclusions
or as part of the spore coat. The pathogenicity of B.t.
to a variety of sensitive insects, such as those in the
orders Lepidoptera and Diptera, is essentially due to

25   this parasporal crystal, which may represent over 20% of
the dry weight of the B.t. cell at the time of
sporulation.

The parasporal crystal is active in the insect
only after ingestion. For instance, after ingestion by a

30   lepidopteran insect, the alkaline pH and proteolytic
enzymes in the mid-gut activate the crystal allowing the
release of the toxic components. These toxic components
poison the mid-gut cells causing the insect to cease
feeding and, eventually, to die. In fact, B.t. has

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proven to be an effective and environmentally safe
insecticide in dealing with lepidopteran pests.

It has been reported that different strains of
B.t. produce serologically different parasporal crystals.
However, one of the predominant crystal forms produced by
many of the B.t. strains is a form known as P-1. P-1 has
a molecular weight of about 130,000-daltons and may also
be present in the spore coat. The genes for the
parasporal crystal P-1 and those of most of the other
protein crystals, have been discovered to reside on any
one of a large number of different plasmids of varying
size in B.t.

2.4   COLEOPTERAN-ACTIVE Bacillus thuringiensis

The first isolation of coleopteran-toxic B.t.
was reported in 1983. (A.Krieg et al. (1983) Z.ang.Ent.
96, 500-508; Ibid. (1984) Anz. Schaedlingskde,
Pflanzenschutz, Umweltschutz 57, 145-150) This strain
makes a single crystal reported to be comprised of
proteins of 68 and 50 kDa (K. Bernhard FEMS Microbiol.
Lett. 33, 261-265 (1986). This strain was given the
designation Bacillus thuringiensis var. tenebrionis. It
was reported that larvae of Lepidoptera and Nematocera
were not sensitive to spores and crystals of this strain.
A similar strain reported by Mycogen Corp. (San Diego,
CA), produces a 64 kDa protein. (C. Herrnstadt et al.
Bio/Technology 4, 305-308 (1986)).

2.5   DELTA-ENDOTOXIN GENE CLONING

Since B.t. toxin genes typically reside on
plasmids and their products have proven to be effective
insecticides which are readily isolated when in

crystalline form or when associated with spore formation, they have been the subject of a great deal of scientific study, particularly with regard to gene isolation and cloning procedures.

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The gene which codes for P-1 has been isolated from B.t. subspecies kurstaki strain HD-1-Dipel, and cloned and expressed in E. coli [Schnepf et al., U.S. Patent 4,467,036]. The protein product, P-1, was determined to be toxic to a lepidopteran insect (tobacco hornworm larvae). The nucleotide sequence of the promoter region and part of the coding region of the crystal protein gene for P-1 have also been determined [H.P. Wong et al., The Journal of Biological Chemistry, Vol. 258, No. 3, pp.1960-1967 (1983)]. The entire nucleotide sequence of this gene has also been determined and the delta-endotoxin protein itself has been expressed in a transformed E. coli strain. [M.J. Adang et al., Gene, Vol, 36, pp.298-300 (1985) and PCT application PCT/US85/01665, for: B.t. Crystal Protein Gene Toxin Segment, (1985)].

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The genes for other delta-endotoxin forms have also been cloned and expressed in E. coli. Recombinant plasmids containing a mosquitocidal delta-endotoxin gene from B.t. var. israelensis was inserted into an E. coli vector. A 26,000-dalton polypeptide was synthesized by E. coli transformed with this vector. This polypeptide was shown to be lethal to insects in the order Diptera (mosquitos). [E.S. Ward et al., FEBS Vol. 175, 2, pp.377-382, 1984]. The nucleotide sequence of the gene coding for this crystal protein was also determined along with the resultant protein sequence [C. Waalwijk et al., Nucleic Acids Research, Vol.13, No. 22, pp.8207-8217, (1985)]. Another B.t. var. israelensis gene encoding a

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130 KDa crystal protein was cloned and used to transform
Bacillus megaterium and Bacillus subtilis. Both B.
megaterium and B. subtilis expressed crystalline
inclusions during sporulation which inclusions were
5 determined to be toxic to the larvae of Aedes aegypti.
[V. Sekar et al., Gene, Vol. 33, pp.151-158, (1985)].

Another delta-endotoxin protein crystal was
derived from B.t. subspecies sotto. The gene coding for
10 this crystalline protein was cloned in a vector and then
expressed in a transformed E. coli. This gene codes for
a 144,000 dalton peptide (934 amino acid residues). The
nucleotide sequence for the gene and the amino acid
sequence of the corresponding protein (as deduced from
15 the DNA sequence) have been reported. [Y. Shibano et
al., Gene, Vol. 34, pp.243-251, (1985)].

It has also been recognized that another major
delta-endotoxin protein is produced by several subspecies
of B.t. [T. Yamamoto, Biochem. and Biophys. Res. Comm.
20 Vol. 103, No. 2, pp.414-421 (1981); T. Yamamoto et al.
Archives of Biochemistry and Biophysics, Vol. 227, No. 1,
pp.233-241 (1983)]. This delta-endotoxin has been
identified as P-2 and isolated from B.t. var. kurstaki
(HD-1). This delta-endotoxin has a molecular weight of
25 approximately 65,000 and is known to be toxic to
lepidopteran and dipteran insects. In contrast, P-1 is
active only against insects of the order Lepidoptera.

To date, although the rare coleopteran active
30 organisms have been isolated neither the toxin protein
nor the gene coding for it have been purified or
sequenced. This fact has rendered it impossible to
provide a means for expressing this uniquely active
delta-endotoxin protein in an organism other than B.t.
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The availability of a cloned gene coding for coleopteran-active protein toxin would enable the enhanced production of this protein in heterologous organisms free of other delta-endotoxins.

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3.0 SUMMARY OF THE INVENTION

This invention relates to a biologically pure culture of a Bacillus thuringiensis strain which has 10. insecticidal activity against insects of the order Coleoptera. This invention also relates to a coleopteran active delta-endotoxin produced by a strain of Bacillus thuringiensis, the DNA sequence for the gene which codes for this protein and novel insecticides incorporating 15 this protein and/or organisms producing it. More specifically, this invention relates to the cloning and transformation of microorganisms with the cryC gene coding for the coleopteran active delta-endotoxin. In addition, this invention is useful in permitting the 20 transformation of a non-sporulating microorganism with the gene coding for the coleopteran active toxin so that it may be produced during virtually all stages of microorganism growth and, thereby, not be limited to production only during a sporulation stage.

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It is, therefore, an object of this invention to provide a biologically pure culture of a Bacillus thuringiensis strain which has insecticidal activity against insects of the order Coleoptera. It is an additional object of this invention to provide a 30 homogeneous coleopteran active protein produced by the isolated gene referred to herein as cryC. This protein may be produced by the process of transforming a microorganism, sporulating or non-sporulating, such as 35 Bacillus megaterium or E. coli or a different strain of

B.t. with the cloned cryC gene. This process, by virtue
of selection of the appropriate host and vector, would
permit high yield production of the delta-endotoxin such
that it is possible to derive a substantially homogeneous
5   preparation of it, i.e. minus any contamination by other
varieties of delta-endotoxins. The coleopteran active
protein and/or the transformed host may be utilized in a
variety of insecticidal compositions.

10   It is further an object of this invention to
provide an organism, other than the native B.t. host,
transformed with the cryC gene. This foreign transformed
host enables the production of the coleopteran active
delta-endotoxin under more desirable and/or selective
15   culturing conditions.

            It is an additional object of this invention to
provide strains of Bacillus thuringiensis which have a
dual activity not found in nature, that is, an
insecticidal activity against insects in the orders
20   Lepidoptera and Coleoptera.

            It is another object of this invention to
provide a DNA probe useful for detecting the presence of
the cryC gene in the various Bacillus thuringiensis
25   strains. This DNA probe also enables the screening of
various strains of B.t. for the possible presence of
related genes coding for proteins sharing a common
homology with the coleopteran active protein and the
isolation of these related genes. It is a further object
30   of this invention to provide a method for controlling
insects of the order Coleoptera with coleopteran active
Bacillus thuringiensis or organisms transformed with the
cryC gene, which renders that strain active against
Coleoptera.
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It is also an object of this invention to provide a method for controlling insects in both the orders Lepidoptera and Coleoptera with transconjugant _Bacillus thuringiensis_ strains which are active against both types of insects, strains which are unknown in the wild. All of the above embodiments of this invention will be described in greater detail in the description of the invention which follows.

4.0   BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows a comparison of the crystal types produced by strain EG2158 and their appearance within the microorganism.

FIGURE 2 is a photograph of a gel electrophoresis showing the respective plasmid arrays of HD-1 and EG2158.

FIGURE 3 is a photograph of a gel electrophoresis showing the respective plasmid arrays of transconjugants harboring coleopteran and lepidopteran active toxin plasmids.

FIGURE 4 is a photograph of a gel electrophoresis showing a comparison of the crystalline proteins from EG2158 to other strains producing the F-1 crystal.

FIGURE 5 is comprised of 5(A), 5(A') and 5(B'). 5(A) is a photograph of a gel electrophoresis of the R-1 and F-1 crystal proteins. 5 (A') and 5(B') are also photographs of electrophoresis gels which show the differential production of 77 and 71 kDa proteins in EG2158 and derivatives of EG2158.

FIGURE 6 is comprised of 6(A) and 6(B), both of which are photographs of a gel electrophoresis showing the productions of the 71 kDa protein in transconjugant strains having the 88-Md plasmid from EG2158.

FIGURE 7 is a restriction map of the recombinant plasmids pEG212 and pEG213 that contain the cloned cryC gene. The location and direction of transcription of the cryC gene are indicated by the large arrow.

FIGURE 8 shows the DNA sequence of the cryC gene (including nucleotides 569 to 2500 which code for the structural toxin protein and nucleotides 2501-2503 code for the "stop" signal) and also the amino acid sequence of the coleopteran toxin encoded by the cryC gene (nucleotides 569-2500).

FIGURE 9 is comprised of 9a and 9b. 9a is a photograph of an ethidium bromide stained Eckhardt gel. The native plasmids that are present in Bacillus thuringiensis strains HD1 and EG2158 are visible illustrating that certain B.t. strains contain several native plasmids. 9b is a photograph of an autoradiogram that was made by hybridizing the radioactively-labeled cloned cryC gene with the plasmids shown in 9a. 9b illustrates that the cloned cryC gene hybridized exclusively to a plasmid of 88 MDa in the coleopteran-toxin strain EG2158 but failed to hybridize to any plasmids in strain HD1, a strain that is not toxic to coleopterans.

FIGURE 10 is a photograph of an SDS/polyacrylamide gel which shows that a recombinant host strain of Bacillus megaterium (EG1314) harboring the

cloned <u>cryC</u> gene synthesizes large quantities of a
protein having a size similar to that of authentic
coleopteran (cry) toxin.

5.0   DESCRIPTION OF THE INVENTION

        Generally stated, the present invention
provides a newly isolated <u>Bacillus</u> <u>thuringiensis</u> strain
which has insecticidal activity against insects of the
order Coleoptera. A biologically pure culture of this
strain has been deposited with the NRRL. Bioassays
described below have confirmed the coleopteran activity
of this strain. This strain of B.t., therefore, is
preferred for use as at least one of the active
ingredients in an insecticide composition useful against
coleopteran insects.

        The present invention further provides for
transconjugant derived <u>Bacillus</u> <u>thuringiensis</u> strains
which have insecticidal activity against both
lepidopteran and coleopteran insects. This dual activity
in <u>B.t.</u> is unknown in the wild. A <u>B.t.</u> strain having
this dual activity would also, therefore, be preferred
for use as at least one of the active ingredients in an
insecticide composition useful against both coleopteran
and lepidopteran insects.

        Additionally, this invention provides,
generally stated, a method for producing <u>Bacillus</u>
<u>thuringiensis</u> strains having insecticidal activity
against both coleopteran and lepidopteran insects
comprising:

        (a)   providing a <u>Bacillus</u> <u>thuringiensis</u> strain
having insecticidal activity against coleopteran insects

conferred by a gene coding for coleopteran active toxin
protein said gene being located on a plasmid said strain
being in admixture with a <u>Bacillus thuringiensis</u> strain
having insecticidal activity against lepidopteran insects
under conditions favoring conjugation and

(b)   isolating from the culture admixture of
step (a) a transconjugant having activity against both
lepidopteran and coleopteran insects.

This method, in a preferred embodiment, also
utilizes intermediate strains to transfer either the
coleopteran or lepidopteran toxin-coding plasmid to
another intermediate recipient strain or directly to the
ultimately desired transconjugant host which already
would contain at least one other of the toxin encoding
plasmids.

The general method described above also
encompasses the embodiment wherein said <u>Bacillus
thuringiensis</u> strain of step (a) having activity against
coleopteran insects additionally has activity against
lepidopteran insects conferred by at least one gene
coding for a lepidoperan-active toxin, whereby said
tansconjugant of step (b) has lepidopteran and
coleopteran activity conferred by at least three toxin
genes.

The general method described above additionally
encompasses the embodiment wherein said <u>Bacillus
thuringiensis</u> strain of step (a) has activity against
lepidopteran insects conferred by more than one toxin
gene, whereby said transconjugant of step (b) has
lepidopteran and coleopteran activity conferred by at
least three toxin genes.

For instance, in the practice of this invention
a strain having coleopteran activity would be provided in
admixture first with a <u>Bacillus</u> <u>thuringiensis</u> strain
whereby said <u>Bacillus</u> <u>thuringiensis</u> strain acquires (by
5   conjugation) the plasmid conferring insecticidal activity
against Coleoptera and then providing the transconjugant
strain in admixture with said <u>Bacillus</u> <u>thuringiensis</u>
having lepidopteran activity under conditions favoring
conjugation whereby said <u>Bacillus</u> <u>thuringiensis</u> strain
10   having lepidopteran activity acquires the plasmid
conferring coleopteran activity by conjugation from said
transconjugant strain.

The present invention also provides for a
15   cloned gene coding for <u>Bacillus</u> <u>thuringiensis</u> coleopteran
active toxin comprising the DNA nucleotide sequence shown
in FIG. 8. This gene (which comprises double stranded
DNA wherein the nucleotide strands have a complementary
base sequence to each other) codes for a protein (or as
also used herein equivalently, polypeptide) having the
20   amino acid sequence of the coléopteran active toxin which
amino acid sequence is shown in FIG. 8. The coleopteran
active toxin encoded by the cloned gene has insecticidal
activity against coleopteran insects.

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Methods of producing the coleopteran active
protein are also provided by this invention. In this
method of production the <u>cryC</u> gene is inserted into a
cloning vector or plasmid which plasmid is then utilized
to transform a selected microorganism.
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The cloning vectors, as described herein, are
generally known in the art and are commercially
available. The choice of a particular plasmid is within
the skill of the art and would be a matter of personal
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choice. Plasmids suitable for use in this invention are,
for instance, pBR322, plasmids derived from B.t., and
plasmids derived from Bacillus and Staphylococcus
microorganisms, preferrably, Bacillus megaterium. Micro-
organisms suitable for use with this invention are both
sporulating and non-sporulating microorganisms such as E.
coli, B.t., and Bacillus megaterium. The microorganisms
utilized are also known in the art and are generally
available. The choice of any particular microorganism
for use in the practice of this invention is also a
matter of individual preference. In a preferred
embodiment of this invention the microorganism would
comprise Bacillus megaterium.

            Generally stated, the coleopteran active toxin
protein can be produced by a transformed organism and
later purified into a homogenous preparation having an
amino acid sequence as shown in FIG. 8. More
specifically, this protein may be produced by
transforming a microorganism with a plasmid containing
the cryC gene, growing the transformed microorganism so
that the protein coded for by the cryC gene is expressed
in the microorganism and by extracting the protein from
the organism with standard protein purification
techniques. It is also within the scope of this
invention that the protein not be separated from the
transformed microorganism but that this organism,
including the expressed coleopteran active protein, be
utilized as or in an insecticidal composition.

            This invention also provides for a novel
insecticide for use against Coleoptera comprising a
mixture of B.t. coleopteran active toxin and a suitable
carrier. The toxin may be contained in the organism or
associated with spores, or be a homogeneous protein

preparation or in a mixture of spores with cultured
transformed organisms. The toxin may also be contained
in a non-sporulating microorganism or a sporulating
microorganism such as Bacillus megaterium or B.t. A
5   suitable carrier may be any one of a number of solids or
liquids known to those of skill in the art.

This invention also comprises the recombinant
vectors or plasmids including the cryC gene and the
10 particular microorganisms which have been transformed
with this gene. In addition, this invention also
provides for oligonucleotide probes for the gene coding
for the coleopteran active delta-endotoxin. All of these
aspects of the inventions are described in detail below
15 and illustrated in the following examples.

5.1   COLEOPTERAN ACTIVE Bacillus thuringiensis

EG2158 is a B.t. strain isolated (deposited and
maintained as a biologically pure culture) from soybean
20 grain dust from Kansas. EG2158 produces two types of
intracellular inclusion during sporulation (FIG. 1): A
somewhat rhomboid crystal (referred to below as R1) and a
flat, diamond-shaped crystal (referred to below as F1).
Bioassays set forth in the Examples below show that
25 sporulated cultures of EG2158 (consisting of a mixture
for spores, R1 and F1 crystals) were toxic to larvae of
the Colorado potato beetle (hereinafter alternatively
referred to as CPB.), Leptinotarsa decemlineata (Say),
but not toxic to lepidopteran larvae of several species
30 (Trichoplusia ni and others).

EG2158 contains a unique plasmid array (FIG. 2)
of 5 plasmids of approximate sizes of 35, 72, 88, 105 and
150 megadaltons (Md).
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Table I below describes which plasmid codes for
a particular toxin.

TABLE I

STRAIN EG2158 COLEOPTERAN ACTIVITY

IS ENCODED BY A TRANSMISSIBLE PLASMID

TOXIN PLASMID	PROPERTIES
150 Md	Encodes "flat diamond" crystal. Loss has no effect on coleopteran activity.
88Md	Encodes rhomboid crystal and coleopteran activity.
	Transfers into B.t. and B. cereus recipient strains. Transconjugant made rhomboid crystal and is toxic to CPB larvae.

Loss of the 150 Md plasmid eliminated production of F1
crystal without affecting toxicity to CPB, while loss of
the 35-Md plasmid had no effect on R1 or F1 production or
toxicity. (Table II)

(Strains of EG2158 and its variants, and all
B.T. and B. Cereus strains were grown for bioassay as
follows: spores were inoculated into 5 mls of M27 broth

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in a 50 ml sterile flask. M27 broth is composed of 33 mM each of $HPO_4^=$ and $H_2PO_4^-$ anions; 98 mM K^+; 0.17% peptone; 0.1% beef extract; 150mM NaCl; 5.5 mM glucose; 330 uM Mg^{++}, 230 uM ca^{++}, and 17 uM Mn^{++} (added as the chloride salts). (As used herein, the letter "u" when used as part of a term of measurement or quantity is synonomous with the prefix "micro".) The cultures were incubated at 30°C with shaking for 3 days, at which time sporulation and crystal formation were complete. Five ul of sterile 1-octanol were added as an anti-foaming agent and the cultures were transferred to sterile plastic tubes, sealed, and stored at 5°C.)

TABLE II

Mortality and leaf consumption by first instar
Colorado Potato Beetle larvae on potato leaf discs
treated with BT

Strain	Number Alive /10 at		Approximate Leaf
	24 h	48 h	Consumption %
(Control)	10	10	90
1 EG2158	10	2	10
2 EG2158 (-150 Md; -flat diamond (F-1))	10	1	15

When EG2158 was grown in mixed culture with other strains
of B.t. or B. cereus, the 105- and 88-Md plasmids were
transmitted, by conjugation, into the other strains.
B.t. or B. cereus stains which acquired the 105-Md
plasmid were not altered detectably (that is, the 105-Md
plasmid is transmissible but otherwise cryptic). B.t. or
B. cereus strains which acquired the 88-Md plasmid were
seen to produce R1 crystals. Therefore, it was
discovered that the 88-Md plasmid is transmissible and
encodes R1 crystals, and yields transconjugant strains
which are R1 producers. The plasmid arrays of some R1-
producing transconjugants are shown in FIG. 3.

TABLE III

TRANSCONJUGANTS HARBORING COLEOPTERAN AND LEPIDOPTERAN-ACTIVE TOXIN PLASMIDS

TRANSCONJUGANT	TOXIN PLASMID (Source strain)	TOXIN PLASMID TARGET INSECTS
HD73-26-46	88 (EG2158)	CPB
HD73-26-50	88 (EG2158)	CPB
	44 (HD-263)	LEP
HD73-26-54	88 (EG2158)	CPB
	61 (HD-617)	LEP
HD73-26-56	88 (EG2158)	CPB
	50 (HD-78)	LEP
	54 (HD-2)	LEP
	75 (HD-2)	LEP
BC569-6-15	88 (EG2158)	CPB
	68 (HD-536)	UNK
HD1-10-1	88 (EG2158)	CPB
HD263-8-5	88 (EG2158)	CPB
	60 (HD-263)	LEP

The 88-Md plasmid was put into recipients of three B.t.
backgrounds (HD-73, HD-1, and HD-263) and one of <u>B.</u>
<u>cereus</u> origin (BC-569). The 88-Md plasmid was shown to
5   coexist with toxin plasmids encoding lepidopteran (P1)
toxin crystals, such as the 44-Md toxin plasmid from HD-
263, and others (See FIG. 3 and Table III).
Transconjugants producing R1 crystals were toxic to CPB,
as was EG2158 (Table IV, A and B) and were also toxic to
10   lepidopteran larvae (Table V, A and B).

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TABLE IV

A. MORTALITY AND LEAF CONSUMPTION BY FIRST INSTAR COLORADO POTATO BEETLE LARVAE ON POTATO LEAF DISCS TREATED WITH Bacillus thuringiensis

Strain	Phenotype	Number Alive/10 at		Approx. Leaf
		24h	48h	Consumption(%)
(Control)		10	9	80
HD73-26-46	Rhomboid$^+$: 88$^+$ <EG2158	5	0	5
HD73-26-47	Rhomboid$^+$: 88$^+$, 105 <EG2158	6	3	5
HD73-26-48	Osp. Rhomboid$^+$: 88$^+$< EG2158	4	2	5
HD73-26-49	Osp. Rhomboid$^+$: 88$^+$, 105 <EG2158	1	0	5
HD73-26-50	Rhomboid$^+$, Pl$^+$: 88$^+$, 105 <EG2158; 44$^+$ <HD263	5	0	5
HD73-26-51	Rhomboid$^+$, [Pl]$^+$: 88$^+$, 105 <EG2158; [54]$^+$ <HD2	2	2	5
HD73-26-52	Rhomboid$^+$, Pl$^+$: 88$^+$, <EG2158; 75$^+$ <HD2	2	2	5
HD73-26-53	Rhomboid$^+$, Pl$^+$: 88$^+$, 105 <EG2158; 75$^+$ <HD2	1	0	5
HD73-26-54	Rhomboid$^+$, Pl$^+$: 88$^+$ <EG2158; 61$^+$ <HD617	5	2	5
HD73-26-55	Rhomboid$^+$, Pl$^+$: 88$^+$, 105 <EG2158; 61$^+$ <HD617	6	1	10
BC569-6-14	Rhomboid$^+$: 88$^+$ <EG2158;	4	1	5
HD73-26	Cry$^-$ control	10	10	60
HD73-26-56	Rhomboid$^+$, Pl$^+$: 88$^+$ <EG2158; 75$^+$, [54]$^+$ <HD2; 50$^+$ <HD78	8	0	20
HD73-26-57	Rhomboid$^+$, Pl$^+$: 88$^+$, 105 <EG2158; 75$^+$, [54]$^+$ <HD2; 50$^+$ <HD78	5	1	5
BC569-6-15	Rhomboid$^+$, Round$^+$: 88$^+$, 105$^+$ <EG2158; 68$^+$ <HD536	7	1	10
HD1-10-1	Rhomboid$^+$: 88$^+$, <EG2158;	8	1	10
HD263-8-5	Rhomboid$^+$, Pl$^+$: 88$^+$, <EG2158; 60$^+$ (native)	5	1	10

TABLE IV

B.   BIOASSAY AGAINST COLORADO POTATO BEETLE

Strain	Dose μg/cup	Mortality % dead	
EG2158	1770	100	
	177	80	
	17.7	20	PLC50 = 55
HD263-8-72	2340	10	
	234	30	
	23.4	20	
HD263-8-73	2450	100	
	245	50	PLC50 = 185
	24.5	10	

Control mortality 10%.

HD263-8-72 contains the same lepidopteran-active plasmids as HD263-8-73, but lacks the 88^{+} Md coleopteran-active plasmid.

TABLE V

A. ACTIVITY OF TRANSCONJUGANTS WITH COLEOPTERAN TOXICITY AGAINST $\underline{OSTRINIA}$ $\underline{NUBILALIS}$ (LEPIDOPTERA)

Liquid Culture	Strain	Donor Plasmid	Pug/ml	No. of Tests	PLC50
MB 96	HD263-8-73	44^+ < HD279	245	3	14.2
AK 3	HD263-8-6	50^+ < HD73	252	3	20.4
AK 4	HD263-8-7	46^+ < HD122A	260	4	20.5
AK 5	HD263-8-8	50^+ < HD119	238	3	13.3
AK 6	HD263-8-9	50^+ < HD78	233	4	11.2
AK 7	HD263-8-10	66^+ < HD588	247	4	27.4
AK 8	HD263-8-11	54, 52 <HD206A	258	4	8.8
AK 9	HD263-8-12	$[47]^+$ <.			
		NB-032786-1C	258	4	10.1

All strains also have the 88^+Md plasmid from EG2158, as well as the 60^+Md P1 toxin plasmid native to HD-263.

B. ACTIVITY OF HD 263-8-5 AGAINST VARIOUS LEPIDOPTERA

Insect	HD 263-8-5		- HD 1-1	
	Dose	% dead	Dose	% dead
$\underline{Heliothis}$ $\underline{virescens}$	25	60	28	80
$\underline{Heliothis}$ $\underline{zea}$	377	30	419	70
$\underline{Spodoptera}$ $\underline{exigua}$	377	10	419	80
$\underline{Lymantria}$ $\underline{dispar}$	38	50	42	80

Dosage is in nanograms crystal protein per diet cup as a surface treatment.

Transconjugants producing both R1 and P1 toxin crystals were shown to be toxic to both CPB and lepidopteran larvae. Their production is described in detail below.

Proteins from the EG2158 crystals, R1 and F1, run on PAGE were determined to be 77, 71 and 31 kDa (see FIG. 4). The R1 crystals were shown to be soluble in 4M NaBr (FIG. 5(A) ("NaBr sup"), leaving the F1 crystals (FIG. 5(A) "NaBr ppt"). This allowed assignment of the 77 kDa and 71 kDa proteins to the R1 crystal (Fig. 5(A)). Recrystallized R1 proteins were toxic to CPB larvae. In certain media and strain backgrounds the 71 kDa protein is produced exclusively (FIG. 5(A') and (B')). FIGURE 5 shows the differential production of 77 and 71 kDa proteins in derivatives of EG2158 on same medium (A') and differential production by one derivative (minus F1) on different media(B'). The extra band at 32 kDa (above F1) is probably a proteolytic fragment of R1.

When the 88-Md plasmid from EG2158 was transferred to other B.t. backgrounds (using the EG2158 culture "O-24" as the source of donor cells), the 71 kDa protein was produced (FIG. 6). These strains are also toxic to CPB. Expression of coleopteran toxin (R1) in kurstaki transconjugants and in B. cereus is undiminished in the presence of other toxin plasmids. The presence of the coleopteran toxin plasmid does not inhibit production of other (e.g. lepidopteran) toxins; nor does it induce the production of toxin by conditional plasmids.

In a preferred embodiment, spores should be included with either EG2158 or other strains harboring the coleopteran toxin plasmid in order to achieve maximum insecticidal activity. These can be spores of the original strain, or spores from another strain.

Southern blotting experiments have shown that a
0.7 kilobase EcoR1 DNA fragment homologous to P1
(lepidopteran) toxin genes does not hybridize to any DNA
sequences in EG2158.

5

5.2   TRANSCONJUGANTS HAVING COLEOPTERAN AND LEPIDOPTERAN ACTIVITY

In a preferred embodiment of this invention
Bacillus thuringiensis strains which have insecticidal
activity against both lepidopteran and coleopteran insects
may be generated by conjugation. To date, Bacillus
thuringiensis strains having this dual activity are
unknown in the wild.

15

Generally stated and as noted above, this
invention also provides a method for producing Bacillus
thuringiensis strains having insecticidal activity against
both coleopteran and lepidopteran insects comprising:

20

(a)   providing a Bacillus thuringiensis strain
having insecticidal activity against coleopteran insects
in admixture with a Bacillus thuringiensis strain having
insecticidal activity against lepidopteran insects under
culture conditions favoring conjugation and

25

(b)   isolating from the culture admixture of
step (a) a transconjugant having activity against both
lepidopteran and coleopteran insects.

30

This method in a preferred embodiment also
utilizes intermediate strains (not having toxin-encoding
plasmids) to transfer either the coleopteran or
lepidopteran toxin-coding plasmid to another intermediate
recipient strain or directly to the ultimately desired

35

transconjugant host (which already would preferrably
contain at least one other of the toxin-encoding
plasmids).

5   More specifically, these transconjugant strains
may all be generated according to the following procedure.

           A BT strain such as EG2158 would be used as a
donor by growing it together with an recipient strain,
10   such as HD73-26. All plasmid transfers would be carried
out by inoculating spores of donor and recipient strains
into M27 broth (or other media suitable for B.t. growth)
and allowing the strains to grow together for 6 or more
hours at 30°C, with gentle shaking. Afterwards, colonies
15   of the recipient strain would be selected for by using
streptomycin-containing plates (in the case of HD73-26,
which is resistant to streptomycin) or would be identified
by random screening. In some cases, nutrient media other
than M27 broth may be used. In this manner, a
20   transconjugant would be created, which would have acquired
plasmids from EG2158. The transconjugant would then be
used as a donor by growing it and a second recipient
strain having toxin plasmids to Lepidoptera together in
liquid broth. The resulting transconjugant would have
25   acquired the 88+Md plasmid from EG2185 which includes the
gene for the Coleoptera active toxin (confirmed by plasmid
array gel electrophoresis).

           The 88-Md coleopteran toxin plasmid of EG2158
30   was transferred by conjugation into HD263-8 (a recipient
BT strain containing a native lepidopteran toxin plasmid,
60 Md in size) to give the transconjugant HD263-8-5
(EG2421), which produces both lepidopteran (P1) and
coleopteran (rhomboid) toxin crystals. In a similar
manner, the 44-Md lepidopteran toxin plasmid of HD279 was
35

transferred to the crystal-negative strain HD73-26 to give
the transconjugant HD73-26-73. HD263-8-5 was then used as
recipient and HD73-26-73 was used as a donor. The
resulting transconjugant, HD263-8-73 (EG2424), has
acquired the 44-Md (P1) toxin plasmid of HD-279, via the
intermediate donor strain HD73-26-73. HD263-8-73 (EG2424)
contains 3 toxin plasmids - the 88-Md coleopteran toxin
plasmid from strain EG2158, and the 60 and 44 Md
lepidopteran toxin plasmids from strains HD263-8 and HD279
respectively. The resulting strain (EG2424) is active
against both Coleoptera (Table IVB) and Lepidoptera (Table
VA), unlike any of the starting strains EG2158, HD263-8,
or HD279. Furthermore, the activity of this strain
(amount of P1 toxin) against Lepidoptera is greater than
that of strain HD263-8-5.

5.3   RECOMBINANT DNA TECHNOLOGY AND GENE EXPRESSION

Generally stated, recombinant DNA technology as
used in the practice of this invention involves insertion
of specific DNA sequences into a DNA vehicle (plasmid or
vector) to form a chimeric DNA molecule which is capable
of replication in a host cell. The inserted DNA sequence
is typically foreign to the recipient host, i.e, the
inserted DNA sequence and the DNA vector are derived from
organisms which do not exchange genetic information in
nature, or the inserted DNA sequence may be wholly or
partially synthetically made. In recent years several
general methods have been developed which enable
construction of recombinant DNA molecules, For example,
U.S. Pat. No. 4,237,224 to Cohen and Boyer describes
production of such recombinant plasmids using restriction
enzymes and methods known as ligation. These recombinant
plasmids are then introduced and replicated in unicellular
organisms by means of transformation. Because of the

general applicability of the techniques described therein,
U.S. Pat. No. 4,237,224 is hereby incorporated by
reference into the present specification.

5   Regardless of the method used for construction,
the recombinant DNA molecule must be compatible with the
host cell, i.e., capable of autonomous replication in the
host cell. The recombinant DNA molecule should also have
a marker function which allows the selection of host cells
10   so transformed by the recombinant DNA molecule. In
addition, if all of the proper replication, transcription
and translation signals are correctly arranged on the
chimeric DNA molecule, the foreign gene will be expressed
in the transformed cells and their progeny.

15

          These different genetic signals and processing
events control many levels of gene expression, i.e., DNA
transcription and messenger RNA translation. Trans-
cription of DNA is dependent upon the presence of a
20   promoter which is a DNA sequence that directs the binding
of RNA polymerase and thereby promotes transcription.

          Translation of messenger RNA (mRNA) in
procaryotes depends upon the presence of the proper
procaryotic signals. Efficient translation of mRNA in
25   procaryotes, such as B.t., requires a ribosome binding
site on the mRNA. This sequence is a short nucleotide
sequence of mRNA that is located before the start codon
(AUG) which encodes the amino-terminal methionine of the
protein. The ribosome binding site is complementary to
30   the 3'-end of the 16S RNA (ribosomal RNA) and probably
promotes binding of mRNA to ribosomes by duplexing with
the mRNA to allow correct positioning of the ribosome
(Roberts and Lauer, 1979, Methods in Enzymology, 68:473).

35

One method widely employed for the cloning of a
particular gene is to prepare a "library" of recombinant
plasmids. Each recombinant plasmid is comprised of a
plasmid vector, which usually confers antibiotic
5   resistance to cells that harbor it, plus a fragment of DNA
from the donor organism, an organism that contains the
gene. The plasmid library is commonly prepared by
digestion of both the plasmid vector and total DNA from
the donor organism with a restriction enzyme, inactivation
10   of the enzyme and ligation of the DNA mixture. The
ligated DNA is a plasmid library. The key feature of this
plasmid library is that it contains many different
recombinant plasmids. It is highly likely that at least
one of the recombinant plasmids in the library will
15   contain a fragment of DNA from the donor organism on which
the gene of interest resides. The plasmid library is
transformed into the cells of a host organism that does
not contain the gene. The host cells are spread on a
selective solid medium, usually one containing an
20   antibiotic, that allows only transformed cells, those
containing recombinant plasmids, to grow into colonies.
Individual transformed host colonies are tested for the
acquisition of the gene from the donor organism. In host
colonies the acquired gene is carried on the recombinant
plasmid.
25

One of the most direct methods of testing for
the acquisition of a gene is to use a gene-specific
hybridization probe, a fragment of DNA that is homologous
to the gene. A characteristic of homologous DNA fragments
30   is that they will bind tightly to each other during
hybridization. Typically a radioactively labeled DNA
probe is used during hybridization so that binding of the
probe to the gene can be easily monitored.

35

A recent advance in molecular biology is the use
of synthetic oligonucleotides as gene-specific probes.
The basis for the use of the oligonucleotides is that in
all biological systems a particular sequence of

5   nucleotides encodes a precise sequence of amino acids.
Conversely if the sequence of amino acids is known for a
particular protein then the nucleotide sequence encoding
the protein can be inferred, although not precisely. In
practice, the partial amino acid sequence of a protein,

10 the product of the gene of interest, is determined by
chemical methods. Based on the protein amino acid
sequence a gene-specific oligonucleotide probe is
synthesized that may be, to varying degrees, homologous to
the gene. Exact homology cannot be guaranteed because

15 knowledge of the amino acid sequence of a protein does not
give exact knowledge of the nucleotide sequence of the
gene encoding the protein. Nevertheless, even though the
homology between the oligonucleotide probe and the gene
may not be precise, hybridization conditions can usually

20 be found that will permit the oligonucleotide probe to
bind specifically to the gene.

Accordingly, in isolating the cryC gene, the
coleopteran toxin was purified from the B. thuringiensis
strain EG2158, and the partial amino acid sequence of the

25 coleopteran toxin was determined. A cryC gene-specific
oligonucleotide probe was synthesized based on the amino
acid sequence of the coleopteran protein. The
oligonucleotide was radioactively labeled and was used in
hybridization experiments to identify transformed host

30 colonies that harbored recombinant plasmids carrying the
cryC gene from the donor B.t. strain.

35

5.4   CLONING OF THE cryC TOXIN GENE FROM BACILLUS THURINGIENSIS STRAIN EG2158

More specifically, in order to clone the cryC

5   toxin gene of this invention, cells of B.t. strain EG2158 were grown in C2 media (1% Glucose, 0.2% Peptone, 0.5% N Z Amine A, 0.2% Yeast Extract, 15mM $(NH_4)_2SO_4$, 23mM KH_2PO_4, 27mM K_2HPO_4, 1mM $MgSO_4.7H_2O$, 600uM $CaCl_2$, 17uM $ZnSO_4.7H_2O$, 17uM $CuSO_4.5H_2O$, 2uM $FeSO_4.7H_2O$) at 30° C

10   until t72 (hours) and spores plus crystals were harvested by centrifugation. The spore/crystal pellet was washed with several changes of 1 M NaCl and then several changes of deionized water. Toxin proteins were solubilized by incubating the spore/crystal preparation in 5% beta-

15   mercaptoethanol, 2% NaDodSO4, 60 mM Tris pH 6.8, 10% glycerol at 70 degrees C. for 7 min., and spores were removed by centrifugation. The supernatant was electrophoresed through polyacrylamide gels containing NaDodSO4 to separate proteins. The gel was stained with

20   Coomassie dye and gel slices containing the coleopteran active protein were cut out with a razor blade. The homogeneous coleopteran active protein preparation was electroeluted from gel slices and, after acetone precipitation, the NH2-terminal amino acid sequence of the coleopteran active protein was determined by automated

25   Edman degradation carried out on an Applied Biosystems Gas Phase Sequenator (model 470A) and analyzed on a DuPont Zorbax C18 column in a Hewlett-Packard HPLC (model 1090) with a 1040 diode array detector. The NH_2-terminal amino acid sequence of the 71 kDA coleopteran toxin has been

30   determined to be:

       1
NH2- ASP GLU ALA LEU THR SER SER THR ASP LYS

35

11
ASP VAL ILE GLN LYS GLY ILE SER VAL VAL

22
ILE ASP LEU LEU

It is significant that Edman sequencing of the
71 kDa coleopteran toxin revealed no NH2-terminal
methionine residue. We believe that the 71 kDa
coleopteran toxin is a processed form of a larger
precursor protein of about 77 kDa. The evidence for this
is as follows. Occasionally on SDS/polyacrylamide gels a
protein of 77 kDa was seen in addition to the 71 kDa
protein from cell extracts of strain EG2158. If the cell
extracts were incubated at 55°C rather than 70°C none of
the 77 kDa protein was seen. At 55°C B.t. proteases would
not be completely inactivated. Protease activity is
probably responsible for processing of the 77 kDa protein
into the 71 kDa form. Since no NH2-terminal methionine
residue was seen in the 71 kDa protein we conclude that
proteases indigenous to B.t. cleave off approximately
5kDa, or 50 amino acids, from the NH2-terminus of the 77
kDa protein to yield the 71 kDa processed protein.

5.5 OLIGONUCLEOTIDE PROBE FOR THE cryC GENE

An oligonucleotide probe encoding amino acids 1
through 22 of the NH2-terminus of the coleopteran active
protein was synthesized on an Applied Biosystems DNA
synthesizer (model 380A). It was recognized that because
of the codon degeneracy (certain amino acids are each
encoded by several slightly different codons) the sequence
of the synthetic oligonucleotide would probably be
different from the actual NH2-terminal sequence of the
cryC gene. However, the fact that the B.t. genome is 68%
A+T and the codon usage information for previously cloned
and sequenced B.t. genes were used in designing an

oligonucleotide probe that would have the highest
probability of matching the actual sequence of the cryC
gene. The oligonucleotide probe was designed to bind only
to the NH2-terminal coding region of the cryC gene. The
5   sequence of the cryC gene-specific oligonucleotide probe
was:

     5'-GAT GAA GCA TTA ACA TCA TCA ACA GAT AAA GAT GTA
ATT CAA AAA GGA ATT TCA GTA GTA ATT GA-3'

10

          In addition to enabling the original isolation
of the cryC gene herein, this DNA probe also comprises
another preferred embodiment of this invention. This DNA
probe permits the screening of any B.t. strain to
15   determine whether the cryC gene (or possibly a related
gene) is naturally present or whether a particular
transformed organism includes the cryC gene. In this
fashion it is also possible to estimate the insecticidal
activity of that strain of B.t. It is also with the scope
20   of this invention that this probe may comprise a smaller
or larger oligonucleotide or another region of the gene.
The probe may be labeled by any number of techniques known
in the art (such as radioactively or enzymatically
labeled) and as described below.

25

30

35

5.6   CONSTRUCTION OF A PLASMID LIBRARY ENRICHED FOR THE cryC GENE

The oligonucleotide probe was used to determine the size of a restriction fragment of B.t. DNA that contained at least the NH2-terminal coding region of the cryC gene. For this determination strain EG2158, the coleopteran toxic strain, was used as a source of DNA. B.t. strain HD1-1, a single colony isolate immediately derived from patent strain HD-1 (U.S.D.A., Brownsville, Texas) was used as a control.

DNA was isolated from the donor strain EG2158 after growth of the cells to mid-log phase at 30° C in LB medium. Cells were harvested by centrifugation, resuspended in 50mM Tris HCl pH 7.8, 10mM EDTA, 1 mg/ml lysozyme and incubated at 37°C for 60 min. Cells were lysed by adding NaDodeSO$_4$ to a final concentration of 0.2%. Cell lysates were extracted twice with an equal volume of phenol and once with an equal volume of chloroform/isoamyl alcohol (24/1). One tenth volume of 3 M NaAcetate and 2 volumes of EtOH were added to the lysates and DNA was extracted by spooling on a glass rod. The spooled DNA was soaked in 66% EtOH for 5 min. and in diethyl-ether for 1 min. The spooled DNA was air dried . and resuspended in deionized water.

Hybridization experiments were performed by digesting total DNA from each of the donor strains with HindIII restriction enzyme, electrophoresing the digested DNA on an agarose gel and transfering the DNA from the agarose gel to a nitrocellulose filter by the blot technique of Southern (J. Molec. Biol. 98:503-517, 1978). The nitrocellulose filter was incubated at 32°C for 16 hrs. in a solution of 3 X SSC (1 X SSC = 0.15M NaCl/0.015

M Sodium Citrate), 0.1 % NaDodeSO$_4$, 200 ug/ml heparin, 10
X Denhardt's (1 X = 0.02% Bovine Serum Albumin/0.02%
Ficoll/0.02% Polyvinyl-Pyrrolidone) containing
approximately 1 ug of the cryC gene-specific
oligonucleotide probe that had been radioactively labeled
with gamma-P32-ATP and T4 kinase. After hybridization the
nitrocellulose filter was washed with 3 X SSC, 0.1 %
NaDodeSo$_4$ at 47°C for one hour and the filter was exposed
to X-ray film. The resulting autoradiogram showed that
the oligonucleotide probe specifically hybridized to a
single Hind III fragment of 2.6 Kb from strain EG2158 but
failed to hybridize to any fragments from the coleopteran
toxin-negative control HD1-1.

A cryC-enriched plasmid library was constructed
by digesting EG2158 total DNA with HindIII,
electrophoresing the digested DNA on an agarose gel and
excising gel slices containing HindIII DNA fragments
ranging in size from approximately 2.0 to 3.0 kb. EG2158
HindIII fragments ranging in size from 2.0 to 3.0 kb were
electroeluted from agarose gel slices, phenol plus
chloroform extracted, ethanol precipitated and ligated
into the HindIII site of plasmid pBR322 that had been
digested with HindIII and treated with alkaline
phosphatase. Alkaline phosphatase greatly increased the
probability that recombinant plasmids were formed
consisting of pBR322 plus a HindIII fragment of EG2158
DNA. The resulting ligation mix consisted of a library of
recombinant plasmids enriched for the cryC toxin gene from
strain EG2158.

5.7 COLONY HYBRIDIZATION AND ISOLATION OF A 2.6 kb HindIII FRAGMENT CONTAINING THE cryC GENE

The cryC gene-enriched plasmid library was
5   transformed into an ampicillin sensitive host strain of E.
coli, HB101 (Bethesda Research Laboratories, Bethesda,
MD.), by the CaCl$_2$ procedure. E. coli strain HB101 does
not synthesize coleopteran toxin protein and, therefore,
it would not be expected to contain the cryC gene. E.
10 coli was used as the host strain because these cells are
easily transformed with recombinant plasmids. All host
cells acquiring a recombinant plasmid would become
ampicillin resistant. After exposure to the recombinant
plasmids the E. coli host cells were spread onto solid
15 medium containing ampicillin and those cells that harbored
a recombinant plasmid were able to grow into colonies. It
was expected that each individual ampicillin resistant
host colony would harbor many identical copies of a
recombinant plasmid comprised of pBR322 plus a unique
20 HindIII fragment from the donor strain EG2158 DNA.
However, the donor strain HindIII fragment in the
recombinant plasmid would differ from one colony to the
next.

25   Approximately two thousand individual ampicillin
resistant colonies were blotted onto nitrocellulose
filters. Replicas of the colonies were saved for later
use as described below. The recombinant plasmids
contained in the colonies were bound to the nitrocellulose
30 filters by treating the colonies with NaOH and NH$_4$Acetate.
The resulting nitrocellulose filters contained an array of
recombinant plasmids each of which was physically
separated from other recombinant plasmids. The
nitrocellulose filters were hybridized at 50°C for 16
35 hours in a solution of 3 X SSC, 200 ug/ml heparin, 0.1%

NaDodeSO$_4$, 10 X Denhardt's and approximately 1 ug of the
cryC gene-specific oligonucleotide probe that had been
radioactively labeled. The filters were washed at 47°C
for one hour in 3 X SSC, 0.1% NaDodeSO$_4$ and were exposed

5   to x-ray film. The resulting autoradiogram showed that
the oligonucleotide probe had hybridized to twelve
different locations on the nitrocellulose filters.

By aligning the autoradiogram with the colony

10   replicas it was possible to identify twelve colonies whose
recombinant plasmids had apparently hybridized with the
oligonucleotide probe.

Plasmids were extracted from each of the twelve

15   colonies. The plasmids were digested with HindIII and
electrophoresed on an agarose gel. The plasmids were
transferred from the agarose gel to a nitrocellulose
filter by the blot procedure of Southern. The
nitrocellulose filter was hybridized with the
radioactively labeled oligonucleotide probe and exposed to

20   x-ray film. The resulting autoradiogram showed that the
oligonucleotide probe hybridized exclusively to a 2.6 kb
HindIII fragment that was contained in only one of the
twelve recombinant plasmids. This recombinant plasmid,
designated pEG212 consisting of pBR322 plus a 2.6 kb

25   HindIII insert from strain EG2158, was selected for
further experimentation and evaluation. The original E.
coli colony harboring pEG212 was designated EG1313.

5.8   LOCATION OF THE cryC GENE ON THE CLONED 2.6 KB

30   HindIII FRAGMENT.

It was likely that the cloned 2.6 kb HindIII
fragment contained at least the NH2-terminal coding region
of the cryC gene. Presence of the cryC gene on this

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fragment was verified using DNA sequencing to search for a
region in the cloned 2.6 kb fragment that encoded the
NH2-terminus of the coleopteran toxin. Since it is time-
consuming to sequence a fragment of DNA longer than two kb
it was necessary to identify a smaller fragment of DNA
within the 2.6 kb fragment that would be expected to
contain the NH$_2$-terminal coding region of the cryC gene.
Accordingly plasmid pEG212 was digested with various
restriction enzymes, digested plasmid was electrophoresed
through an agarose gel and plasmid restriction fragments
were blotted from the gel to a nitrocellulose filter.
Hybridization of the filter with the radioactively labeled
oligonucleotide probe revealed that the probe specifically
hybridized to a 1.0 kb Pst 1 - EcoRI restriction fragment
of DNA from pEG212. Therefore it was expected that the
1.0 kb Pst1 - EcoRI fragment would contain at least the
NH2-terminal coding region of the cryC gene.

The 1.0 kb fragment was subcloned from pEG212
into the DNA sequencing vectors mp18 and mp19 (Bethesda
Research Laboratories, Bethesda MD). DNA sequencing of
the 1.0 kb fragment revealed that it contained a region of
DNA that encoded the NH2-terminal amino acids, with a few
amino acid exceptions noted below, of the 70 kDa
coleopteran toxin. This conclusively demonstrated that
the cloned 2.6 kb HindIII fragment from the donor strain
EG2158 contained the cryC gene.

5.9   DNA SEQUENCE OF THE CLONED cryC GENE

A restriction map of the cloned 2.6 kb HindIII
fragment contained in plasmid pEG212 is shown in FIG. 7.
The large arrow indicates a region of approximately 2.0 kb
that was assumed to encode the entire coleopteran toxin.
In order to determine the complete sequence of the cryC

gene the entire 2.6 kb HindIII fragment in plasmid pEG212
was subcloned into the sequencing vectors mp18 and mp19.
FIGURE 8 shows the DNA sequence of the 2.6 kb HindIII
fragment beginning with the first nucleotide in the

5   HindIII site that is upstream from the cryC gene as shown
on plasmid pEG212, FIG. 7. At nucleotide 569 (FIG. 8) a
long open reading frame (protein coding region) was found
beginning with an NH2-terminal methionine codon.
Preceding the methionine codon is a ribosome binding site

10   (GGAGGA) at nucleotide 557. At nucleotide 728, fifty-
three amino acids downstream from the NH2-terminal
methionine codon, the coding region for the NH2-terminus
of the 71 kDa coleopteran toxin begins. This region
encodes several aspartate and threonine residues that were

15   determined by sequential Edman degradation of the 71 kDa
protein to be threonine and aspartate residues,
respectively (compare the NH2-terminal sequence of the 71
kDa protein with the coding region of the cryC gene
beginning at nucleotide 728). These discrepancies are due
to the difficulty in accurately determining the NH2-

20   terminal amino acid sequence of proteins. Because of the
precision with which DNA sequences can be determined the
correct amino acid sequence for the coleopteran toxin must
be as shown in FIGURE 8.

25
        As indicated in FIG. 8 the NH2-terminal coding
region for the 71 kDa protein begins 53 amino acid
residues downstream from the NH2-terminal methionine
codon. Fifty-three amino acids are equivalent to
approximately 6 kDa, precisely the difference in size

30   between the 71 kDa protein and its assumed precursor of 77
kDa. Therefore, DNA sequencing of the cloned cryC gene
clearly shows that the gene encodes a protein (77 kDa)
that is subsequently proteolytically processed to yield a
protein (71 kDa) that is 6 kDa smaller.

35

5.10   USE OF THE CLONED cryC GENE AS A SPECIFIC
           HYBRIDIZATION PROBE.

5.10.1   IDENTIFICATION OF NATIVE B.t. PLASMIDS
              CONTAINING cryC GENES.

One advantage of a cloned DNA sequence is that
it can be used to identify related DNA sequences in
uncharacterized samples of DNA. In the case of the cryC
gene it is now possible that the cloned gene can be used
to detect the presence of a cryC gene in a strain of B.t.

In order to determine whether the cloned cryC
gene could be used to detect the presence and locations of
a cryC gene in a native B.t. host strain the following
procedure was carried out. B.t. strains HD1-1, and EG2158
were lysed according to the procedure of Eckhardt
(Eckhardt, T. (1978) Plasmid 1:584-588) and the lysates
were electrophoresed through agarose gels. This procedure
allowed the separation by size of all plasmids contained
in a particular strain. The separated plasmids were
transferred from the agarose gel to a nitrocellulose
filter by the blot procedure of Southern. The
nitrocellulose filter was hybridized with the
radioactively labeled 2.6kb HindIII (cryC gene) fragment.
Autoradiography of the nitrocellulose filter revealed that
the cryC gene fragment hybridized exclusively to one
plasmid of approximately 88 MDa in the coleopteran toxin-
producing strain EG2158 (Figure 9). The cloned cryC gene
did not hybridize to any plasmids in the coleopteran
toxin-negative strain HD1-1. Therefore, this experiment
demonstrated that the cloned cryC gene can be used in a
direct manner to identify native plasmids containing cryC
genes in B.t. strains. DNA hybridization with the cloned
cryC gene allowed direct identification of a single

plasmid carrying a cryC gene out of many such plasmids
existing in strains of B.t.

5.11   TRANSFORMATION OF THE cryC GENE INTO HETEROLOGOUS
        MICROORGANISMS

5

        The cryC gene can be inserted in any appropriate
plasmid which may then be utilized to transform an
appropriate microorganism. It is clearly within the scope
of this invention that microorganisms other than B.t. may
10
be transformed by incorporation of the cryC gene i.e.,
generally stated, organisms from the genera Bacillus and
Escherichia. Preferred for use with this invention is the
organism Bacillus megaterium.

15

        The microorganisms so transformed will
preferably produce the Coleoptera active protein toxin in
quantities that are far in excess of the quantity of this
toxin produced in a B.t. natural host strain. The
coleopteran active toxin produced by a transformed
20
organism is preferably the only delta-endotoxin produced
by that organism. In this manner, the organism itself may
be utilized alone or as part of an insecticidal
composition. Since coleopteran active toxic would
preferably be the only delta-endotoxin produced by the
25
organism, it is a straightforward process to purify the
coleopteran active protein from other cellular material by
methods known in the art such as Renografin density
gradients.

30
5.12   TRANSFORMATION OF THE cryC GENE INTO PLANTS

        It is also within the scope of this invention
that the cryC gene (FIG. 8) be inserted directly into a

35

plant so that the plant itself produces the cryC
coleopteran active toxin.

     Genetic engineering of plants may be
accomplished by introducing the desired DNA containing the
cryC gene into plant tissues or cells using DNA molecules
of a variety of forms and origins. These include, but are
not limited to: DNA molecules derived from naturally
occurring plant vectors such as the Ti plasmid from
Agrobacterium tumefaciens or plant pathogens such as DNA
viruses like Cauliflower Mosaic Virus (CaMV) or
Geminiviruses, RNA viruses, and viroids; DNA molecules
derived from unstable plant genome components like
extrachromosomal DNA elements in organelles (e.g.,
chloroplasts or mitochondria), or nuclearly encoded
controlling elements; DNA molecules from stable plant
genome components (e.g., origins of replication and other
DNA sequences which allow introduced DNA to integrate into
the organellar or nuclear genomes and to replicate
normally, to autonomously replicate, to segregate normally
during cell division and sexual reproduction of the plant
and to be inherited in succeeding generations of plants).

     DNA containing the cryC gene may be delivered
into the plant cells or tissues directly by infectious
plasmids, such as the Ti plasmid, viruses or
microorganisms like A. tumefaciens, the use of liposomes,
microinjection by mechanical methods and by whole
chromosomes or chromosome fragments.

5.13   PRODUCTS AND FORMULATIONS
       INCORPORATING THE COLEOPTERAN ACTIVE TOXIN

     The coleopteran delta-endotoxin coded for by the
cryC gene is a potent insecticidal compound with activity

against coleopteran insects. It is, therefore, within the
scope of the invention that this protein toxin be utilized
as an insecticide (the active ingredient) alone,
preferably in homogenous or pure form and having the amino
acid sequence of FIG. 8, or as included within or in
association with the B.t. strain EG2158 or with a
transformed microorganism which expresses a cloned cryC
gene or in a mixture of B.t. transconjugants or other
transformed sporulating microorganisms containing cryC
gene protein product toxin with spores or otherwise.

        The compositions of the invention containing at
least the cryC protein toxin are applied to the
appropriate Coleoptera (or Lepidoptera) habitat at an
insecticidally effective amount which will vary depending
on such factors as, for example, the specific coleopteran
(or also lepidopteran if a dual active transconjugant is
used) insects to be controlled, the specific plant to be
treated and the method of applying the insecticidally
active compositions.

        Target crops (potential habitats for Coleoptera
and Lepidoptera) protected by the present invention
comprise e.g. the following species of plants: cereals
(such as wheat, barley, rye, oats, rice, sorghum and
related crops), beets, leguminous plants, oil plants
(such as poppy, olives, and sunflowers) cucumber plants,
fibre plants, citrus fruit, vegetables, deciduous trees
and conifers.

        The preferred insecticide formulations are made
by mixing EG2158 alone or any mutant, recombinant or
genetically engineered derivative thereof, in an effective
amount or the coleopteran active toxin alone or
incorporated in or associated with another organism (i.e.

a transformed organism or transconjugant), with the
desired carrier. The formulations may be administered as
a dust or as a suspension in oil (vegetable or mineral) or
water, a wettable powder or in any other material suitable
for agricultural application, using the appropriate
carrier adjuvants. Suitable carriers can be solid or
liquid and correspond to the substances ordinarily
employed in formulation technology, e.g., natural or
regenerated mineral substances, solvents, dispersants,
wetting agents, tackifiers, binders or fertilizers.

Generally stated, the preferred compositions
usually contain 0.1 to 99%, preferably 1 to 50%, of the
insecticidal microorganism such as Bacillus thuringiensis,
or combination thereof with other active ingredients, 1 to
99.9% of a solid or liquid adjuvant, and 0 to 25%
preferably 0.1 to 20%, of a surfactant.

The formulations containing a solid or liquid
adjuvant, are prepared in known manner, e.g., by
homogenously mixing and/or grinding the active ingredients
with extenders, e.g., solvents, solid carriers, and in
some cases surface active compounds (surfactants).

Suitable liquid carriers are vegetable oils,
such as coconut oil or soybean oil, mineral oils or water.
The solid carriers used, e.g., for dusts and dispersable
powders, are normally natural mineral fibers such as
calcite, talcum, kaolin, or attapulgite. In order to
improve the physical properties it is also possible to add
highly dispersed silicic acid or highly dispersed
absorbent polymers. Suitable granulated adsorptive
carriers are porous types, for example pumice, broken
brick, seplolite or bentonite. Suitable nonsorbent
carriers are materials such as silicate or sand. In

addition, a great number of pregranulated materials or inorganic or organic mixtures can be used, e.g., especially dolomite or pulverized plant residues.

5   Depending on the nature of the active ingredients to be formulated, suitable surface-active compounds are non-ionic, cationic and/or anionic surfactants having good emulsifying, dispersing and wetting properties. The term "surfactants" will also be

10   understood as comprising mixtures or surfactants.

Suitable anionic surfactants can be both water-soluble soaps and water-soluble synthetic surface active compounds.

15   Suitable soaps are the alkali metal salts, alkaline earth metal salts or unsubstituted ammonium salts of higher fatty acids (C_{10}-C_{11}), e.g., the sodium or potassium salts of oleic or stearic acid, or natural fatty acid mixtures which can be obtained, e.g., from coconut

20   oil or tallow oil. Further stable surfactants are also the fatty acid methyltaurin salts as well as modified and unmodified phospholipids.

25   More frequently, however, so-called synthetic surfactants are used, especially fatty sulfonates, fatty sulfates, sulfonated benzimidazole derivatives or alkylarylsulfonates.

30   The fatty sulfonates or sulfates are usually in the forms of alkali metal salts, alkaline earth metal salts or unsubstituted ammonium salts and generally contain a C_6-C_{22} alkyl, e.g., the sodium or calcium salt of dodecylsulfate, or of a mixture of fatty alcohol sulfates, obtained from fatty acids. These compounds also

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comprise the salts of sulfonic acid esters and sulfonic acids of fatty alcohol/ethylene oxide adducts. The sulfonated benzimidazole derivatives preferably contain 2 sulfonic acid groups and one fatty acid radical containing

5 about 8 to 22 carbon atoms. Examples of alkylarylsulfonates are the sodium, calcium or triethanolamine salts of dodecylbenzenesulfonic acid, dibutynaphthalenesulfonic acid, or of a naphthalenesulfonic acid/formaldehyde condensation

10 product. Also suitable are corresponding phosphates, e.g., salts of the phosphoric acid ester of an adduct of p-nonylphenol with 4 to 14 moles of ethylene oxide.

Nonionic surfactants are preferbly polyglycol

15 ether derivative or aliphatic or cycloaliphatic alcohol or saturated or unsaturated fatty acids and alkylphenols, said derivative containing 3 to 10 glycol ether groups and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon moiety and 6 to 18 carbon atoms in the alkyl moiety of the

20 alkylphenols.

Other suitable non-ionic surfactants are the water soluble adducts of polyethylene oxide with alkypropylene glycol, ethylenediaminopolypropylene glycol

25 and alkylpolypropylene glycol contain 1 to 10 carbon atoms in the alkyl chain, which aducts contain 20 to 250 ethylene glycol ether groups and 10 to 100 propylene glycol ether groups.

Representative examples of non-ionic surfactants

30 are nonylphenolpolyethoxyethanols, castor oil, glycol ethers, polypropylene/polyethylene oxide adducts, tributylphenoxypolyethoxyethanol, ethylene glycol and octylphenoxypolyethoxynethanol. Fatty acid esters of

35

polyoxyethylene sorbitan, such as polyoxyethylene sorbitan trioleate, are also suitable non-ionic surfactants.

Cationic surfactants are preferably quaternary ammonium salts which contain, as substituents on the nitrogen, at least one C_8-C_{22} alkyl radical and, as further substituents, lower unsubstituted or halogenated alkyl benzyl, or hydroxylated lower alkyl radicals. The salts are preferably in the form of halides, methyl sulfates or ethylsulfates, e.g., stearyltrimethylammonium chloride.

6.0 EXAMPLES

The insecticidal activity of B.t., transformed or non-transformed _Bacillus megaterium_ and of transformed _Escherichia coli_ was determined by including various amounts of these microorganisms in a test diet which was fed to insects. After feeding, insect mortality was measured.

Specifically, these bioassays involved growing the microorganism to stationary phase in liquid culture or on solid agar base media for two days at 30°C. For _E. coli_ harboring plasmids the media was LB containing 40 ug/ml ampicillin. For _B. megaterium_ harboring plasmids the media was DS containing 10 ug/ml tetracycline. The microorganisms were harvested from the solid medium by scraping with a spatula. The wet weight of the harvested bacteria was determined and bacterial cells were resuspended to a known concentration in deionized water. 100 ul of the bacterial cell suspension was topically applied to 3 ml of a solid agar-based artificial diet in a feeding cup. The top surface area of the diet was 600 square millimeters. One neonate larva of Colorado potato

beetle (CPB) was placed in each feed cup and mortality was
scored after seven days.

6.1   EXAMPLE 1 - TRANSFORMATION OF THE
      cryC GENE INTO BACILLUS MEGATERIUM

The purpose of this example was to determine
whether the cloned cryC gene would be expressed in
Bacillus strains. Plasmid pEG212 (containing the cryC
gene) will replicate only in gram-negative strains such as
E. coli. In order to test for the expression of the
cloned cryC gene in a Bacillus strain it was first
necessary to construct a recombinant plasmid that
contained the cryC gene and that was capable of
replicating in Bacillus. A Bacillus-E. coli "shuttle
vector" that contained the cryC gene was constructed. The
term "shuttle vector" indicates that the plasmid is
capable of replication both in Bacillus and in E. coli.
The E. coli - Bacillus shuttle vector was constructed by
digestion of the Bacillus plasmid pBC16 (tetracycline
resistance) with Sph1, ligation of the digested plasmid
into the Sph1 site of pEG212 (ampicillin resistance) and
transformation of E. coli to ampicillin and tetracycline
resistance.

One tet and amp resistant E. coli transformant
harbored a plasmid (designated pEG213) that was composed
of pBC16 inserted into the Sph1 site of pEG212 (Figure 7).
Figure 7 shows the restriction map of plasmid pEG213. The
boxed areas denote plasmid vector DNA. The open box is
pBR322 DNA (E. coli replication) and the cross-hatched box
is pBC16 DNA (Bacillus replication). The horizontal line
is cloned DNA from strain EG2158. The large arrow denotes
the coding region of the cryC gene. pEG213 was
transformed into Bacillus megaterium (ATCC deposit number

35985) and one tetracycline resistant transformant harboring pEG213 (designated strain EG1314) was chosen for further study.

This example determined if the cloned cryC gene was expressed in the recombinant B. megaterium strain EG1314 (pEG213). Gene expression was measured by the technique of NadodeSO4/polyacrylamide gel electrophoresis. Generally, the technique involved preparation of cell lysates, electrophoresis of cell lysates through a NadodeSO4/polyacrylamide gel and staining of the gel to permit visualization of proteins.

Specifically, the technique was carried out as follows: B. megaterium cells were grown on DS plates containing 10ug/ml tetracycline for 48 hr. at 30°C. B. thuringiensis strain EG2158 was grown similarly to B. megaterium except the DS plates contained no tetracycline. After this period almost all cells had entered the stationary phase of growth. Cells were harvested with a spatula and resuspended in deionized water. A portion of the cell suspension was mixed 1:2 vol:vol with preheated (70°C) gel loading buffer (5% Beta-mercaptoethanol, 2% NaDodeSO4, 60 mM Tris pH 6.8, 10% glycerol) and incubated at 70°C for 7 min. The suspension was centrifuged briefly, after centrifugation the supernatant was immediately loaded onto an NadodeSO4/polyacrylamide gel and the proteins in the supernatant were resolved by gel electrophoresis according to the method of Laemmli. (1973) J. of Mol. Bio., 80:576-599) The proteins in the gel were visualized by staining the gel with Coomassie dye.

Figure 10 is a photograph of an NadodeSO4/polyacrylamide gel that had been prepared as

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described above. The lane labeled STND in FIG. 10
contained protein molecular weight standards. Numbers to
the right of the gel indicate protein sizes in kilodaltons
(kDa). The lane labeled EG2158 contained extracts of that
5   B.t. strain. The major protein band that corresponded to
the coleopteran toxin protein is indicated by an arrow.
The lane labeled CRY contained a portion of the purified
coleopteran toxin protein. The coleopteran toxin protein
was purified as described above.

10
        The lanes labeled EG1311 and EG1314 in Fig. 10
contained extracts of these B. megaterium strains
harboring pBC16 and pEG213(cryC) respectively. A
comparison of lanes EG1311 and EG1314 showed that extracts
15 of strain EG1314(pEG213) contained a major protein that
corresponded in size to that of the coleopteran toxin
protein. This protein was not present in extracts of
strain EG1311(pBC16). This demonstrates that B.
megaterium harboring the cloned cryC gene synthesized high
levels of the coleopteran toxin protein. In addition, when
20 viewed under the light microscope the cells of strain
EG1314 appeared to contain phase-bright protein inclusion
bodies characteristic of crystal toxins.

25 6.2 BIOASSAY OF THE EXPRESSION PRODUCT OF
        THE CLONED cryC GENE IN B. MEGATERIUM

        B. megaterium strain EG1314 (pEG213-cryC) was
tested for toxicity against Colorado potato beetle (CPB).
30 A cell suspension was prepared by growing strains EG1311
(pBC16-negative control) and EG1314 on solid DS medium
containing 10 ug/ml tetracycline for 48 hours at 30 C.
Cells were harvested with a spatula and cells were
resuspended in deionized water. The bacterial cell
35 suspensions were topically applied to 3 ml of a solid

agar-base artificial diet in a feeding cup. One neonate
larvae of CPB was added per cup and mortality was scored
after seven days. (TABLE VI)

5

TABLE VI

Dose-mg cells/ml	CPB larvae # dead/total
EG1311 (pBC16-control) - 0.2 mg/cup	3/50
EG1314 (pEG213-cryC) - 0.2 mg/cup	49/50

15

7.0 DEPOSIT OF MICROORGANISMS

It is within the scope of this invention that a
wide variety of both sporulating and nonsporulating
microorganisms may be transformed with the cryC gene as
described herein. Exemplary of the microorganisms which
may be engineered are those from the genera Bacillus and
Escherichia. Preferred for use with this invention is the
organism Bacillus megaterium. In addition, the following
Bacillus thuringiensis, Bacillus megaterium and E. coli
strains which are also preferred for use with this
invention and which carry the listed plasmids have been
deposited with the Agricultural Research Culture
Collection (NRRL), Peoria, IL and have been assigned the
listed accession numbers:

30

35

B. thuringiensis strain	Plasmids	Accession Numbers
EG2158	Several naturally occurring, including the 88-Md coleopteran toxin plasmid	B-18213
EG2421 (HD263-8-5)	Several naturally occurring plasmids, including a 60-Md lepidopteran toxin plasmid, as well as the 88-Md toxin plasmid from EG2158	B-18212
EG2424 (HD263-8-73)	Several naturally occurring plasmids, including a 60-Md lepi-dopteran toxin plasmid, plus the 88-Md toxin plasmid from EG2158 and a 44-Md lepidopteran toxin plasmid from HD-279.	B-18214

B. megaterium	Plasmid	Accession Numbers
EG1314	pEG213	B-18210

E. coli	Plasmid	Accession Numbers
EG1313	pEG212	B-18211

The present invention is not to be limited in scope by the microorganisms deposited, since the deposited embodiments are each intended as a single illustration of one aspect of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings.

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Internatio

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page ___52___, line __1-24__ of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3]   SEE ATTACHED

Name of depositary institution [4]

Agricultural Research Culture Collection
International Depository Authority

Address of depositary institution (including postal code and country) [4]

1815 N. University Street
Peoria, Illinois 61604
U.S.A.

Date of deposit [5]	Accession Number [6]
SEE ATTACHED SHEET	SEE ATTACHED SHEET

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the International application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

[X] The date of receipt (from the applicant) by the International Bureau [10]

W88

_____
(Authorized Officer)

Form PCT/RO/134 (January 1981)

MICROORGANISMS

A. IDENTIFICATION OF DEPOSIT:

	Date of Deposit	Accession Number
B. megaterium (EG1314)	April 28, 1987	B-18210
E. coli (EG1313)	April 28, 1987	B-18211
B. thuringiensis (HD263-8-5)	April 29, 1987	B-18212
B. thuringiensis (EG2158)	April 29, 1987	B-18213
B. thuringiensis (HD263-8-73)	April 29, 1987	B-18214

What is claimed is:

1.   A gene for <u>Bacillus</u> <u>thuringiensis</u> delta-endotoxin
having the DNA sequence of FIGURE 8 nucleotides 569 to
2500 or any portions or derivatives thereof.

2.   The gene of claim 1 wherein said gene codes for a
protein having the amino acid sequence of FIGURE 8.

3.   The gene of claim 2 wherein said protein has
insecticidal activity.

4.   The gene of claim 3 wherein said insecticidal
activity is effective against Coleoptera.

5.   The gene of claim 1 wherein said DNA sequence is
inserted into a recombinant plasmid.

6.   The gene of claim 5 wherein said plasmid is comprised
of DNA from at least two different species of
microorganisms after insertion of said DNA sequence.

7.   The gene of claim 5 wherein said plasmid is comprised
of DNA from at least two different subspecies of the same
species of microorganism after insertion of said DNA
sequence.

8.   The gene of claim 1 wherein said DNA sequence is
attached to its native promoter DNA sequence.

9.   The gene of claim 1 wherein said DNA sequence is
attached to a foreign promoter DNA sequence.

10. A protein having the amino acid sequence of FIGURE 8
or any portions or derivatives thereof.

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11.   The protein of claim 10 wherein said protein has
insecticidal activity.

12.   The protein of claim 11 wherein said insecticidal
5   activity is effective against Coleoptera.

13.   The protein of claim 10 wherein said protein is
produced by the process comprising:

10   a) transforming a microorganism with the gene
              of FIGURE 8 nucleotides 569 to 2503;

              b) growing said transformed microorganism
              whereby the protein encoded by said gene of
              step a) is expressed in said microorganism and
15

              c) extracting and separating said protein
              expressed in step b) from said organism.

20 14.   The protein of claim 13 wherein said gene of step a)
is located on a plasmid.

15.   The protein of claim 14 wherein said plasmid is
comprised of DNA from at least two different species of
microorganisms when including said gene.
25

16.   The protein of claim 14 wherein said plasmid is
comprised of DNA from at least two different subspecies
of microorganism when including said gene.

30
17.   The protein of claim 13 wherein said protein is
expressed in a non-sporulating microorganism.

18.   The protein of claim 15 and 16 wherein the gene is
controlled by its native promoter.
35

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19. The protein of claim 15 and 16 wherein the gene is controlled by a foreign promoter.

20. The protein of claim 19 wherein said protein is expressed in a sporulating microorganism.

21. The protein of claim 18 wherein the protein is expressed during non-sporulating growth phases of said microorganism.

22. The protein of claim 13 wherein said protein is extracted in step c) by lysis of said microorganism.

23. The protein of claim 10 wherein said protein is in substantially pure form.

24. A method for producing <u>Bacillus thuringiensis</u> delta-endotoxin comprising:

    a)   inserting into a plasmid a gene for said delta-endotoxin having the DNA sequence of FIGURE 8 nucleotides 569 to 2503;

    b)   transforming a microorganism with the plasmid of step a) and

    c)   growing the transformed microorganisms of step b) whereby said delta-endotoxin is expressed in said microorganisms.

25. The method of claim 24 wherein said gene codes for a protein having the amino acid sequence of FIGURE 8.

26. The gene of claim 24 wherein said plasmid is comprised of DNA of at least two different species of

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microorganism after insertion of said delta-endotoxin
gene.

27.   The method of claim 24 wherein said plasmid is
comprised of DNA from at least two different subspecies
of the same species of microorganism after insertion of
said delta-endotoxin gene.

28.   The method of claim 24 wherein said DNA sequence is
attached to its native promoter DNA sequence.

29.   The gene of claim 24 wherein said DNA sequence is
attached to a foreign promoter DNA sequence.

30.   The method of claim 24 wherein said microorganism is
a non-sporulating microorganism.

31.   The method of claim 24 wherein said microorganism is
a sporulating microorganism.

32.   The method of claim 30 wherein the delta-endotoxin
is expressed during non-sporulating growth phases of said
microorganism.

33.   The method of claim 24 wherein said delta-endotoxin
is extracted from the microorganism by lysis of said
microorganism.

34.   An insecticide suitable for use against Coleoptera
comprising a mixture of a <u>Bacillus</u> <u>thuringiensis</u> delta-
endotoxin and a suitable carrier.

35.   The insecticide of claim 34 wherein the delta-
endotoxin is associated with <u>Bacillus</u> <u>thuringiensis</u>
spores.

36. The insecticide of claim 34 wherein the delta-endotoxin is a homogeneous protein preparation.

37. The insecticide of claim 34 wherein the delta-endotoxin is contained in a mixture of <u>Bacillus</u> <u>thuringiensis</u> spores and cultured <u>Bacillus</u> <u>thuringiensis</u> organisms.

38. The insecticide of claim 34 wherein the delta-endotoxin is associated with a non-sporulating microorganism.

39. The insecticide of claim 34 wherein the delta-endotoxin is associated with a sporulating microorganism

40. The insecticide of claim 34 wherein the carrier is a liquid carrier.

41. The insecticide of claim 40 wherein the liquid carrier contains one or more surfactants.

42. The insecticide of claim 34 wherein the carrier is a solid carrier.

43. The insecticide of claim 42 wherein the solid carrier is selected from the group consisting of calcite, talcum, koalin, attapulgite, silicate, sand, dolomite, and pulverized plant residue.

44. The insecticide of claim 42 wherein the solid carrier is a granulated adsorptive carrier.

45. The insecticide of claim 44 wherein the granulated adsorptive carrier is selected from the group consisting of pumice, broken brick, seplolite, and bentonite.

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46. The insecticide of claim 42 further comprising a surfactant.

47. A recombinant vector containing the DNA sequence of claim 1.

48. A non-sporulating microorganism containing the DNA sequence of claim 1.

49. The non-sporulating microorganism of claim 48 wherein said microorganism is E. coli.

50. A sporulating microorganism containing the DNA sequence of claim 1.

51. A microorganism containing the DNA sequence of claim 1 selected from the group consisting of Bacillus, Escherichia and Cyanobacteria.

52. A Escherichia coli bacterium deposited with NRRL and assigned Accession No. B-18211, or a mutant, recombinant, or genetically engineered derivative thereof.

53. A Bacillus megaterium bacterium deposited with NRRL and assigned Accession No. B-18210, or a mutant, recombinant, or genetically engineered derivative thereof.

54. A Bacillus thuringiensis bacterium deposited with NRRL and assigned Accession No. B-18213, or a mutant, recombinant, or genetically engineered derivative thereof.

55.   An oligonucleotide probe for the gene coding for
coleopteran active delta-endotoxin comprising the
sequence:

5   5'-GAT GAA GCA TTA ACA TCA TCA ACA GAT AAA GAT GTA ATT
CAA AAA GGA ATT TCA GTA GTA ATT GA-3'

      or derivative thereof.

10   56.   The oligonucleotide probe of claim 55 wherein said
probe is labeled.

57.   The oligonucleotide probe of claim 56 wherein said
probe is labeled with a radioactive label.

15
58.   A coleopteran toxin gene-specific probe comprised of
the DNA sequence of claim 1 wherein said DNA or a portion
or derivative thereof is labeled.

20   59.   The probe of Claim 58 wherein said DNA or portion or
derivative thereof is labeled with a radioactive label.

60.   A plant transformed with the DNA sequence of
claim 1.

25
61.   The plant of claim 60 wherein the plant produces the
delta-endotoxin or portion thereof of FIGURE 8.

62.   A Bacillus thuringiensis bacteria having activity
against insects selected from the orders consisting of
30   Lepidoptera and Coleoptera.

63.   The Bacillus thuringiensis bacteria of claim 62
wherein said bacteria is deposited with the NRRL and has
been assigned Accession No. B-18212, or a mutant,
35

recombinant, or genetically engineered derivative thereof.

64. The _Bacillus thuringiensis_ bacteria of claim 62 wherein said bacteria is deposited with the NRRL and has been assigned Accession No. B-18214 or a mutant, recombinant, or genetically engineered derivative thereof.

65. A method of controlling insects of the order Coleoptera comprising applying to a Coleoptera habitat an effective amount of _Bacillus thuringiensis_ of claim 54.

66. A method of controlling insects of the order Lepidoptera and Coleoptera comprising applying to a Lepidoptera and Coleoptera Habitat an effective amount of the _Bacillus thuringiensis_ of claim 62.

67. A method of controlling insects of the order Coleoptera comprising applying to a Coleoptera habitat an effective amount of _Escherichia coli_ of claim 52.

68. A method of controlling insects of the order Coleoptera comprising applying to a Coleoptera habitat an effective amount of the _Bacillus megaterium_ of claim 53.

69. A method for producing a _Bacillus thuringiensis_ having insecticidal activity against both coleopteran and lepidopteran insects comprising:

    (a) providing a _Bacillus thuringiensis_ strain having insecticidal activity against coleopteran insects conferred by a gene coding for coleopteran active toxin protein said gene being located on a plasmid said _Bacillus thuringiensis_ strain being in admixture with

another Bacillus thuringiensis strain having
insecticidal activity against lepidopteran insects under
conditions favoring conjugation and

5   (b)   isolating from the culture admixture of
step (a) a transconjugant having activity against both
lepidopteran and coleopteran insects.

70.   The method of claim 69 further comprising providing
the Bacillus thuringiensis strain having coleopteran
activity in admixture first with an intermediate
recipient Bacillus strain whereby said intermediate
recipient Bacillus strain acquires by conjugation the
plasmid conferring insecticidal activity against
Coleoptera and then providing the transconjugant strain
in admixture with said Bacillus thuringiensis having
lepidopteran activity under conditions favoring
conjugation whereby said Bacillus thuringiensis strain
having lepidopteran activity aquires the plasmid
confering coleopteran activity from said transconjugant
strain.

71.   The method of claim 69 wherein said Bacillus
thuringiensis strain of step (a) having activity against
coleopteran insects additionally has activity against
lepidopteran insects conferred by at least one gene
coding for a lepidoperan-active toxin, whereby said
tansconjugant of step (b) has lepidopteran and
coleopteran activity conferred by at least three toxin
genes.

72.   The method of claim 69 wherein said Bacillus
thuringiensis strain of step (a) has activity against
lepidopteran insects conferred by more than one toxin
gene, whereby said transconjugant of step (b) has

-64-

lepidopteran and coleopteran activity conferred by at
least three toxin genes.

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FIGURE I

COLEOPTERAN-TOXIC
<u>B</u>. <u>THURINGIENSIS</u>
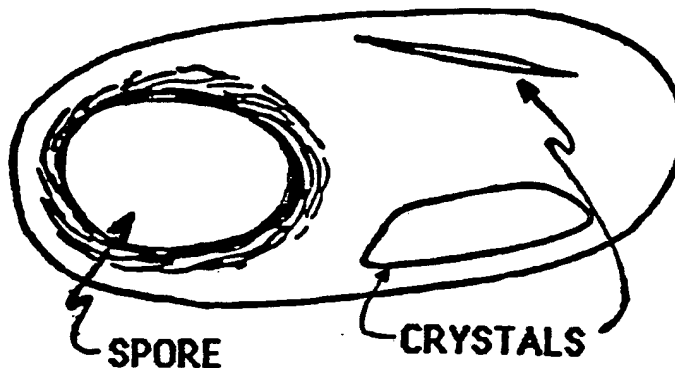
SPORE   CRYSTALS

Strain: EG2158

Source: Soybean grain dust, Kansas

Crystal
Phenotype: Two crystals per sporangium

1.   Rhomboid crystal   (R-1)
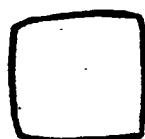
top view   side view

2.   Flat diamond-shaped crystal   (F-1)

top view   side view

EG 2158
Toxicity: Colorado Potato Beetle larvae.

Non-toxic to lepidopteran larvae.
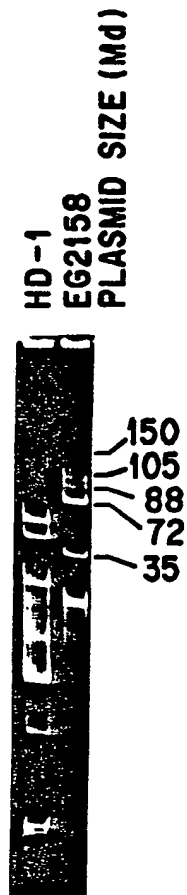
PLASMID ARRAYS OF
HD-1 AND EG2158

FIG. 2
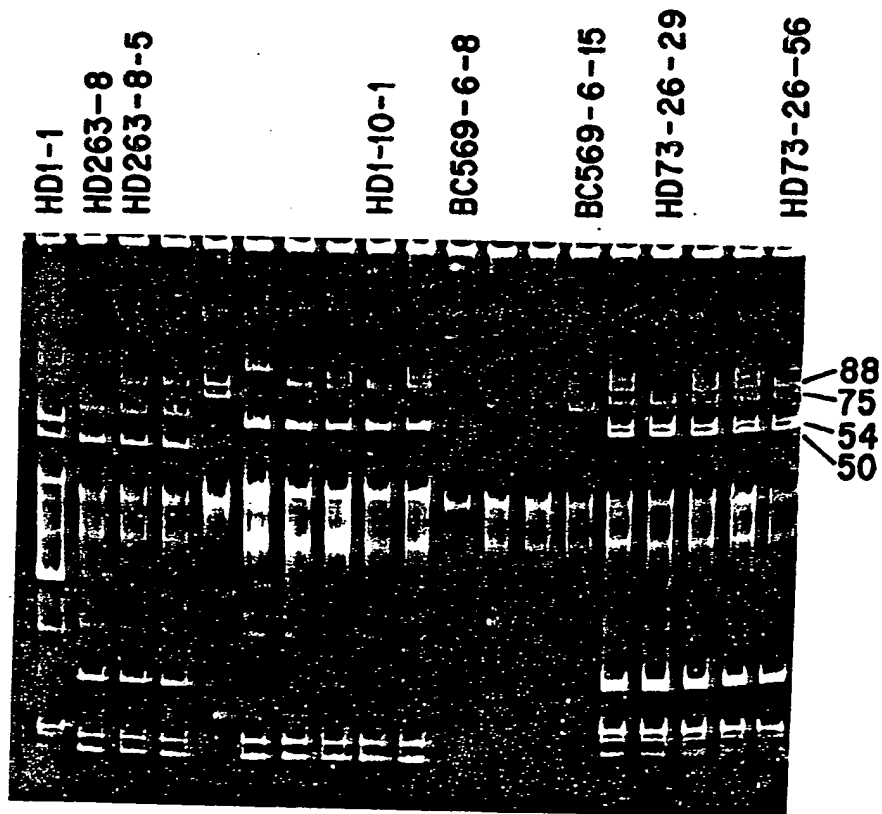
PLASMID ARRAYS OF SOME TRANSCONJUGANTS
HARBORING THE 88 Md COLEOPTERAN TOXIN
PLASMID

FIG. 3

4/13
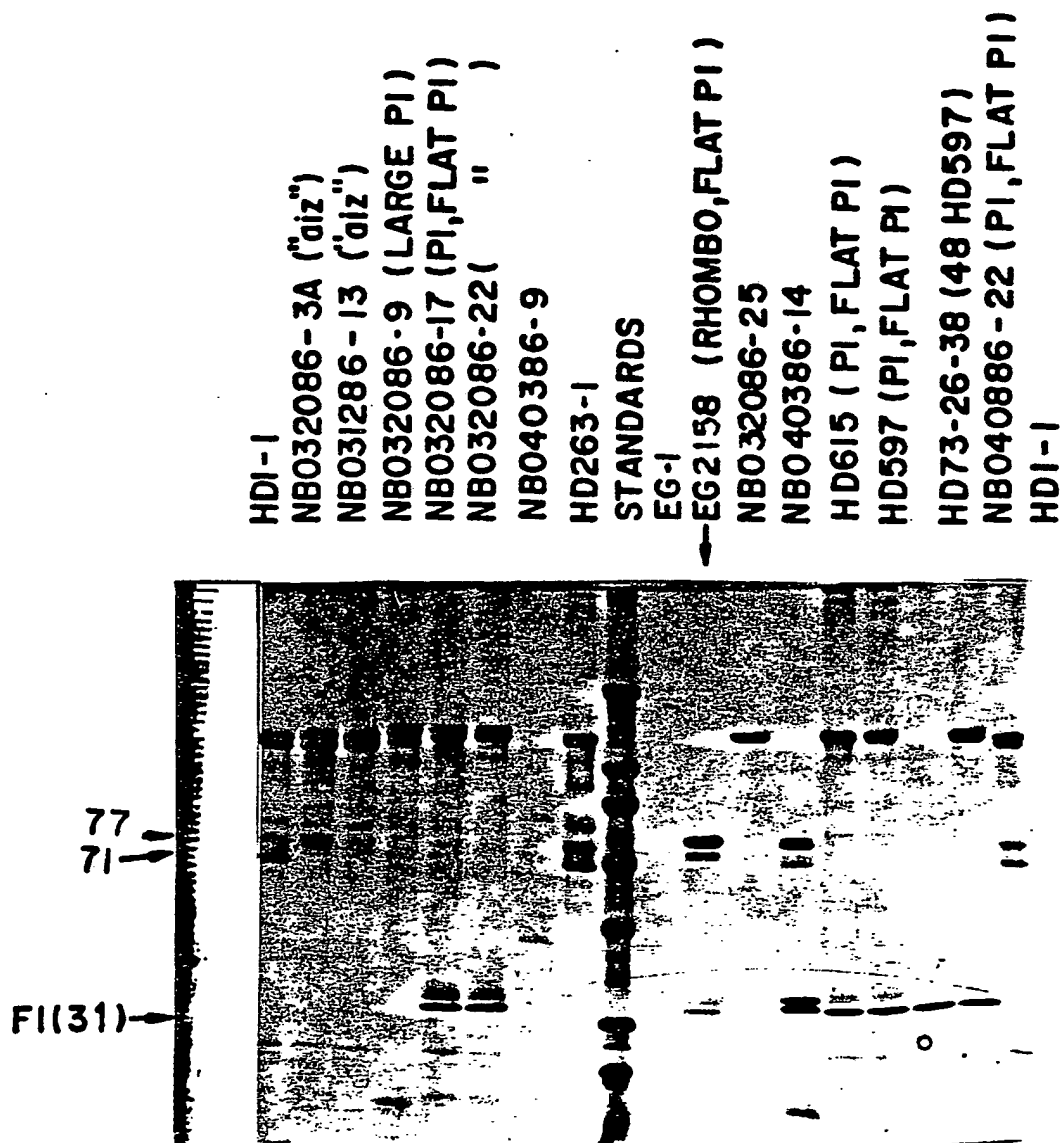
ORIGINAL ISOLATE OF COLEOPTERAN-TOXIC BT;
COMPARISON TO OTHER STRAINS PRODUCING 'FLAT PI'
(FI) CRYSTALS.
ARROWS INDICATE PROTEINS MADE BY EG2158.
NB NUMBER INDICATES NEW BACILLUS ISOLATE.

FIG.4

FIG. 5

A   STANDARDS
EG 2158

NaBr sup

NaBr ppt

A'   EG2158
NB032086-12B (-150,- F-1)
NB032086-12C (-35, n.c.)
EG2158 (REPURIF.)

B'   NB032086-12B (C2)
" (M-27)
" (Med B)

6/13
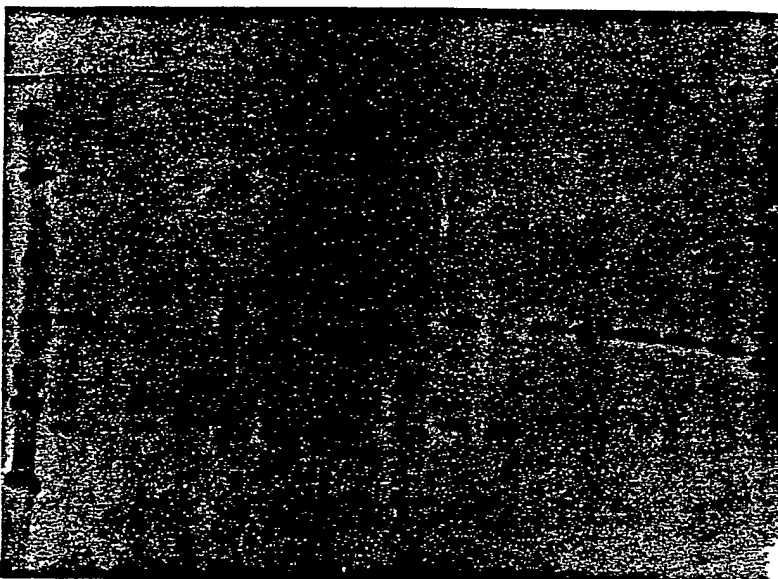
EG2158
HD73-26-46 (88<0-24)
HD73-26-47 (88,105<0-24)
HD73-26-48 (88<0-24,OLIGOSPOROGENOUS)
HD73-26-49 (88,105<0-24, OLIGOSP.)
BC569-6-14 (88 <0-24)
HD73-26-10 (44<HD263)
HD73-26-50 (44<HD263; 88, 105<0-24)
HD73-26-19 ([54], 75 <HD2)
HD73-26-51 ([54]<HD2; 88, 105<0-24)
HD73-26-18 (75<HD2)
HD73-26-52 (75<HD2; 88<0-24)
HD73-26-53 (75<HD2; 88, 105<0-24)
HD73-26-40 (61<HD617)
HD73-26-54 (61<HD617; 88<0-24)
STANDARDS
HD73-26-55 (61<HD617; 88,105<0-24)

HD73-26-56 ([54], 75<HD2; 50<HD78; 88<0-24)
HD536 (3X LOADED)
BC569-6-15 (68<HD536; 88<0-24)
BC569-6-8 (68<HD536; 3X LOADED)
HD1-10-1 (88<0-24)
HD263-8-5 (60; 88<0-24)

FIG. 6

FIGURE 7
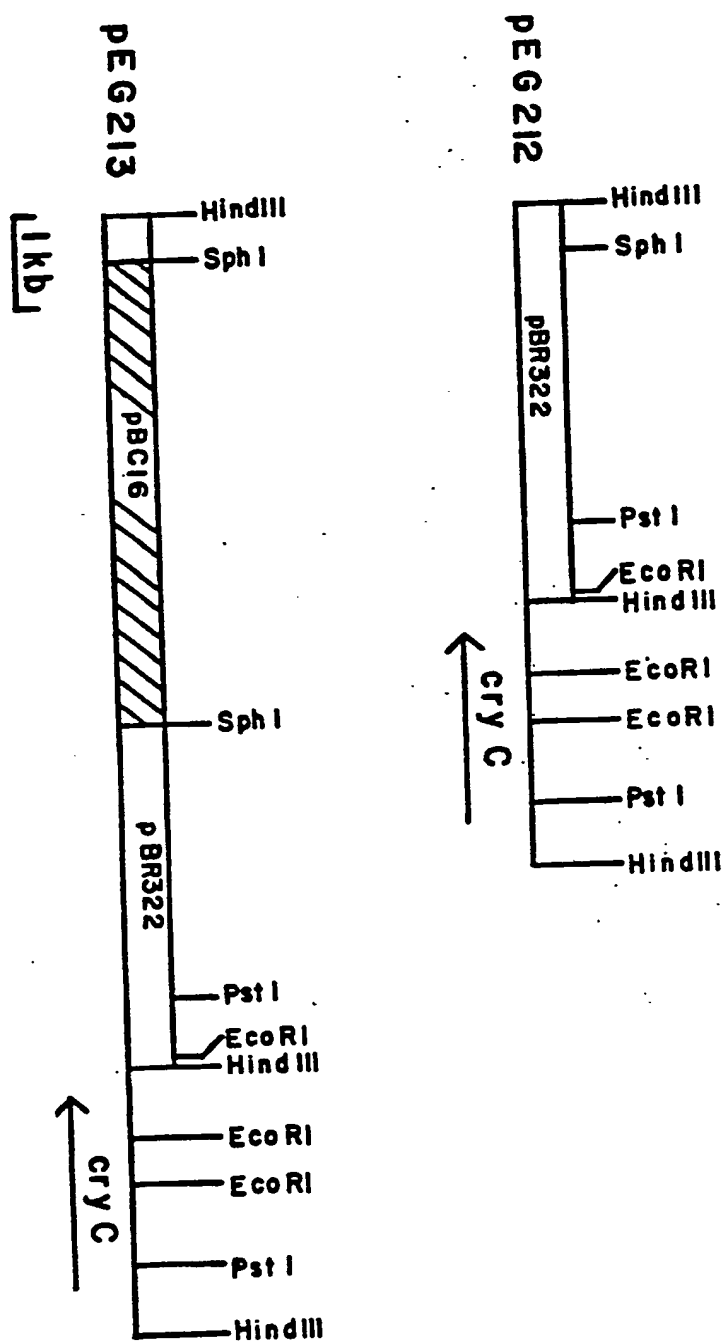
cryC

FIG. 8-1

```
           10          20          30          40          50          60
AAGCTTAATTAAAGATAATATCTTTGAATTGTAACGCCCCTCAAAAGTAAGAACTACAAA


           70          80          90         100         110         120
AAAAGAATACGTTATATAGAAATATGTTTGAACCTTCTTCAGATTACAAATATATTCGGA


          130         140         150         160         170         180
CGGACTCTACCTCAAATGCTTATCTAACTATAGAATGACATACAAGCACAACCTTGAAAA


          190         200         210         220         230         240
TTTGAAAATATAACTACCAATGAACTTGTTCATGTGAATTATCGCTGTATTTAATTTTCT


          250         260         270         280         290         300
CAATTCAATATATAATATGCCAATACATTGTTACAAGTAGAAATTAAGACACCCTTGATA


          310         320         330         340         350         360
GCCTTACTATACCTAACATGATGTAGTATTAAATGAATATGTAAATATATTTATGATAAG


          370         380         390         400         410         420
AAGCGACTTATTTATAATCATTACATATTTTCTATTGGAATGATTAAGATTCCAATAGA


          430         440         450         460         470         480
ATAGTGTATAAATTATTTATCTTGAAAGGAGGGATGCCTAAAAACGAAGAACATTAAAAA


          490         500         510         520         530         540
CATATATTTGCACCGTCTAATGGATTTATGAAAAATCATTTATCAGTTTGAAAATTATG


          550         560         570         580         590         600
TATTATGATAAGAAAGGGAGGAAGAAAAATGAATCCGAACAATCGAAGTGAACATGATAC
                                        MetAsnProAsnAsnArgSerGluHisAspTh


          610         620         630         640         650         660
AATAAAAACTACTGAAAATAATGAGGTGCCAACTAACCATGTTCAATATCCTTTAGCGGA
rIleLysThrThrGluAsnAsnGluValProThrAsnHisValGlnTyrProLeuAlaGl


          670         680         690         700         710         720
AACTCCAAATCCAACACTAGAAGATTTAAATTATAAAGAGTTTTTAAGAATGACTGCAGA
uThrProAsnProThrLeuGluAspLeuAsnTyrLysGluPheLeuArgMetThrAlaAs


          730         740         750         760         770         780
TAATAATACGGAAGCACTAGATAGCTCTACAACAAAAGATGTCATTCAAAAAGGCATTTC
pAsnAsnThrGluAlaLeuAspSerSerThrThrLysAspValIleGlnLysGlyIleSe


          790         800         810         820         830         840
CGTAGTAGGTGATCTCCTAGGCGTAGTAGGTTTCCCGTTTGGTGGAGCGCTTGTTTCGTT
rValValGlyAspLeuLeuGlyValValGlyPheProPheGlyGlyAlaLeuValSerPh


          850         860         870         880         890         900
TTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTATGGA
eTyrThrAsnPheLeuAsnThrIleTrpProSerGluAspProTrpLysAlaPheMetGl
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FIG. 8-2

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         910        920        930        940        950        960
ACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTCTTGC
uGlnValGluAlaLeuMetAspGlnLysIleAlaAspTyrAlaLysAsnLysAlaLeuAl

         970        980        990        1000       1010       1020
AGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGCA
aGluLeuGlnGlyLeuGlnAsnAsnValGluAspTyrValSerAlaLeuSerSerTrpGl

         1030       1040       1050       1060       1070       1080
AAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGTTTC
nLysAsnProValSerSerArgAsnProHisSerGlnGlyArgIleArgGluLeuPheSe

         1090       1100       1110       1120       1130       1140
TCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACGAGGT
rGlnAlaGluSerHisPheArgAsnSerMetProSerPheAlaIleSerGlyTyrGluVa

         1150       1160       1170       1180       1190       1200
TCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAGACGC
lLeuPheLeuThrThrTyrAlaGlnAlaAlaAsnThrHisLeuPheLeuLeuLysAspAl

         1210       1220       1230       1240       1250       1260
TCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATAAAAG
aGlnIleTyrGlyGluGluTrpGlyTyrGluLysGluAspIleAlaGluPheTyrLysAr

         1270       1280       1290       1300       1310       1320
ACAACTAAAACTTACGCAAGAATATACTGACCATTGTGTCAAATGGTATAATGTTGGATT
gGlnLeuLysLeuThrGlnGluTyrThrAspHisCysValLysTrpTyrAsnValGlyLe

         1330       1340       1350       1360       1370       1380
AGATAAATTAAGAGGTTCATCTTATGAATCTTGGGTAAACTTTAACCGTTATCGCAGAGA
uAspLysLeuArgGlySerSerTyrGluSerTrpValAsnPheAsnArgTyrArgArgGl

         1390       1400       1410       1420       1430       1440
GATGACATTAACAGTATTAGATTTAATTGCACTATTTCCATTGTATGATGTTCGGCTATA
uMetThrLeuThrValLeuAspLeuIleAlaLeuPheProLeuTyrAspValArgLeuTy

         1450       1460       1470       1480       1490       1500
CCCAAAAGAAGTTAAAACCGAATTAACAAGAGACGTTTTAACAGATCCAATTGTCGGAGT
rProLysGluValLysThrGluLeuThrArgAspValLeuThrAspProIleValGlyVa

         1510       1520       1530       1540       1550       1560
CAACAACCTTAGGGGCTATGGAACAACCTTCTCTAATATAGAAAATTATATTCGAAAACC
lAsnAsnLeuArgGlyTyrGlyThrThrPheSerAsnIleGluAsnTyrIleArgLysPr

         1570       1580       1590       1600       1610       1620
ACATCTATTTGACTATCTGCATAGAATTCAATTTCACACGCGGTTCCAACCAGGATATTA
oHisLeuPheAspTyrLeuHisArgIleGlnPheHisThrArgPheGlnProGlyTyrTy

         1630       1640       1650       1660       1670       1680
TGGAAATGACTCTTTCAATTATTGGTCCGGTAATTATGTTTCAACTAGACCAAGCATAGG
rGlyAsnAspSerPheAsnTyrTrpSerGlyAsnTyrValSerThrArgProSerIleGl

         1690       1700       1710       1720       1730       1740
ATCAAATGATATAATCACATCTCCATTCTATGGAAATAAATCCAGTGAACCTGTACAAAA
ySerAsnAspIleIleThrSerProPheTyrGlyAsnLysSerSerGluProValGlnAs

         1750       1760       1770       1780       1790       1800
TTTAGAATTTAATGGAGAAAAAGTCTATAGAGCCGTAGCAAATACAAATCTTGCGGTCTG
nLeuGluPheAsnGlyGluLysValTyrArgAlaValAlaAsnThrAsnLeuAlaValTr
```

FIG. 8-3

```
        1810      1820      1830      1840      1850      1860
GCCGTCCGCTGTATATTCAGGTGTTACAAAAGTGGAATTTAGCCAATATAATGATCAAAC
pProSerAlaValTyrSerGlyValThrLysValGluPheSerGlnTyrAsnAspGlnTh

        1870      1880      1890      1900      1910      1920
AGATGAAGCAAGTACACAAACGTACGACTCAAAAAGAAATGTTGGCGCGGTCAGCTGGGA
rAspGluAlaSerThrGlnThrTyrAspSerLysArgAsnValGlyAlaValSerTrpAs

        1930      1940      1950      1960      1970      1980
TTCTATCGATCAATTGCCTCCAGAAACAACAGATGAACCTCTAGAAAAGGGATATAGCCA
pSerIleAspGlnLeuProProGluThrThrAspGluProLeuGluLysGlyTyrSerHi

        1990      2000      2010      2020      2030      2040
TCAACTCAATTATGTAATGTGCTTTTTAATGCAGGGTAGTAGAGGAACAATCCCAGTGTT
sGlnLeuAsnTyrValMetCysPheLeuMetGlnGlySerArgGlyThrIleProValLe

        2050      2060      2070      2080      2090      2100
AACTTGGACACATAAAAGTGTAGACTTTTTTAACATGATTGATTCGAAAAAAATTACACA
uThrTrpThrHisLysSerValAspPhePheAsnMetIleAspSerLysLysIleThrGl

        2110      2120      2130      2140      2150      2160
ACTTCCGTTAGTAAAGGCATATAAGTTACAATCTGGTGCTTCCGTTGTCGCAGGTCCTAG
nLeuProLeuValLysAlaTyrLysLeuGlnSerGlyAlaSerValValAlaGlyProAr

        2170      2180      2190      2200      2210      2220
GTTTACAGGAGGAGATATCATTCAATGCACAGAAAATGGAAGTGCGGCAACTATTTACGT
gPheThrGlyGlyAspIleIleGlnCysThrGluAsnGlySerAlaAlaThrIleTyrVa

        2230      2240      2250      2260      2270      2280
TACACCGGATGTGTCGTACTCTCAAAAATATCGAGCTAGAATTCATTATGCTTCTACATC
lThrProAspValSerTyrSerGlnLysTyrArgAlaArgIleHisTyrAlaSerThrSe

        2290      2300      2310      2320      2330      2340
TCAGATAACATTTACACTCAGTTTAGACGGGGCACCATTTAATCAATACTATTTCGATAA
rGlnIleThrPheThrLeuSerLeuAspGlyAlaProPheAsnGlnTyrTyrPheAspLy

        2350      2360      2370      2380      2390      2400
AACGATAAATAAAGGAGACACATTAACGTATAATTCATTTAATTTAGCAAGTTTCAGCAC
sThrIleAsnLysGlyAspThrLeuThrTyrAsnSerPheAsnLeuAlaSerPheSerTh

        2410      2420      2430      2440      2450      2460
ACCATTCGAATTATCAGGGAATAACTTACAAATAGGCGTCACAGGATTAAGTGCTGGAGA
rProPheGluLeuSerGlyAsnAsnLeuGlnIleGlyValThrGlyLeuSerAlaGlyAs

        2470      2480      2490      2500      2510      2520
TAAAGTTTATATAGACAAAATTGAATTTATTCCAGTGAATTAAATTAACTAGAAAGTAAA
pLysValTyrIleAspLysIleGluPheIleProValAsn

        2530      2540      2550      2560      2570      2580
GAAGTAGTGACCATCTATGATAGTAAGCAAAGGATAAAAAAATGAGTTCATAAAATGAAT

        2590      2600      2610      2620      2630      2640
AACATAGTGTTCTTCAACTTTCGCTTTTTGAAGGTAGATGAAGAACACTATTTTTATTTT

        2650      2660      2670      2680      2690      2700
CAAAATGAAGGAAGTTTTAAATATGTAATCATTTAAAGGGAACAATGAAAGTAGGAAATA
```

FIG. 8-4

```
     2710        2720        2730        2740          2750          2760
AGTCATTATCTATAACAAAATAACATTTTTATATAGCCAGAAATGAATTATAATATTAAT

     2770        2780        2790        2800          2810          2820
CTTTTCTAAATTGACGTTTTTCTAAACGTTCTATAGCTTCAAGACGCTTAGAATCATCAA

     2830        2840        2850        2860          2870          2880
TATTTGTATACAGAGCTGTTGTTTCCATCGAGTTATGTCCCATTTGATTCGCTAATAGAA

     2890        2900        2910        2920          2930          2940
CAAGATCTTTATTTTCGTTATAATGATTGGTTGCATAAGTATGGCGTAATTTATGAGGGC

     2950        2960        2970        2980
TTTTCTTTTCATCAAAAGCCCTCGTGTATTTCTCTGTAAG
```

Fig. 9
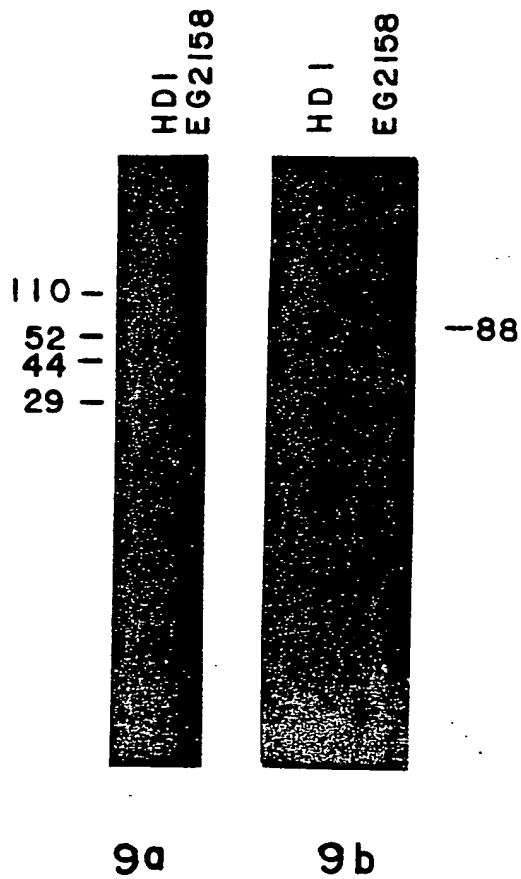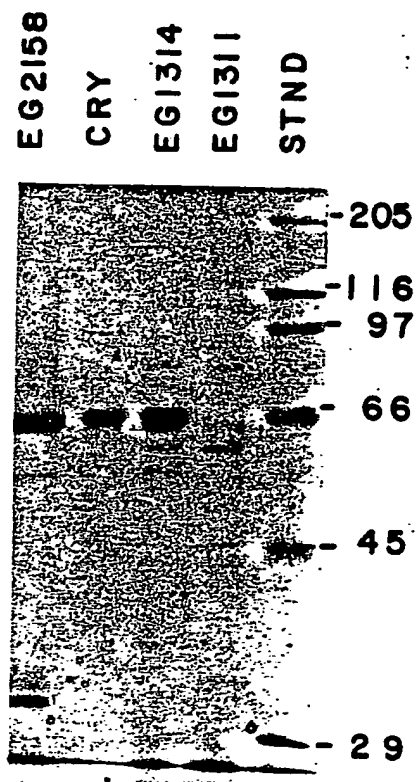
9a   9b

Fig. 10

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (4) C12P 21/00, C12N 15/00, C12N 1/20, A61K 31/52

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
U.S.	424/93; 536/27, 47/58, 800/1 435/68,70,172.1,172.3,253,320,882,884

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

Computer Search Chemical Abstracts, Biological Abstracts:
Bacillus, thuringiensis, toxin, endotoxin, plasmid, conjugat!,
coleopteran, truncat!, delet!

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	BIOTECHNOLOGY, Volume 4, issued 1986 April (Clinton, Iowa, USA) (C. HERRNSTADT ET AL.), "A new strain of Bacillus thuringiensis with activity against coleopteran insects" See pages 305-308.	1-72
Y, P	BIOTECHNOLOGY, Volume 6, issued 1988, January (Clinton, Iowa, USA), (S.A. McPHERSON ET AL.), "Characterization of the coleopteran specific protein gene of Bacillus thuringiensis var. tenebrionis" See pages 61-66.	1-72

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 JULY 1988	2 2 AUG 1988
International Searching Authority	Signature of Authorized Officer
ISA/US	ROBIN LYN TESKIN

	III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)	
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	GENE, volume 36, issued 1985, (Amsterdam, The Netherlands) (M.J.ADANG ET AL.), "Characterized full-length and truncated plasmid clones of the crystal protein of Bacillus thuringiensis subsp. kurstaki HD-73 and their toxicity to Manduca sexta" See pages 289-300.	1-61
Y	GENE, Volume 33, issued 1985, (Amsterdam, The Netherlands) (V. SEKAR ET AL.), "Molecular cloning of the delta-endotoxin gene of Bacillus thuringiensis var. israelensis" See pages 151-158.	1-61
Y	GENE, Volume 34, issued 1985, (Amsterdam, The Netherlands) (Y. SHIBANO ET AL), "Nucleotide sequence coding for the insecticidal fragment of the Bacillus thuringiensis crystal protein" See pages 243-251.	1-61
Y	US, A, 4,448,885 (SCHNEPF ET AL.) issued 15 May 1984, See entire document.	1-61
Y,P	CHEMICAL ABSTRACTS, Volume 107, issued 03 August 1987 (Columbus, Ohio, U.S.A.), (D. KARAMATA ET AL.), "Hybrid Bacillus thuringiensis producing δ-endotoxins of kurstaki and tenebrionsis strains" See pages 275-276 Abstract No. 35171m.	62-72
Y	PLASMID, Volume 11, issued January, 1984, (New York, New York U.S.A), (J.M. GONZALEZ ET AL.), "A large transmissable plasmid is required for crystal toxin production in Bacillus thuringiensis variety israelensis" See pages 28-38.	62-72
Y	MOL. GEN. GENET. Volume 191, issued 1983 (Berlin, West Germany) (A. KLIER ET AL.), "Mating between Bacillus subtilis and Bacillus thuringiensis and transfer of cloned crystal genes" See pages 257-262.	62-72

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	PROC. NATL. ACAD. SCI. Volume 79, ·issued November 1982, (Washington, D.C. U.S.A.), (J.M. GONZALEZ ET AL). "Transfer of Bacillus thuringiensis plasmids coding for δ -endotoxin among strains of B. thuringiensis and B. cereus" See pages 6951-6955.	62-72
Y	FEBS LETTERS, Volume 158 Number 1, issued July 1983, (Amsterdam, The Netherlands) (E.S. WARD ET AL), "Assigment of the δ -endotoxin gene of Bacillus thuringiensis var. israelensis to a specific plasmid by curing analysis" See pages 45-49.	62-72
Y ·	U.S., A, 4,609,550 (FITZ-JAMES) issued 02 September 1986, See the entire document.	21,30,38, 48-49
Y	BIOTECHNOLOGY, Volume 5, issued 1987, (Clinton, Iowa (D.A. FISCHHOFF ET AL), "Insect tolerant transgenic· tomato plants" See pages 807-813.	60-61
Y	E.P.A., 0,142,924, (ADANG ET AL), issued 29 May 1985 see the entire document.	60-61

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	
Y	Z. AGNEW. ENT., Volume 96, issued 1983 (Munich, West Germany) (A. KRIEG ET AL.), "Bacillus thuringiensis var. tenebrionsis a new pathotype effective against larvae of coleoptera" (translation from German) See entire document.	1-72

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers_____ , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers_____ , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.